Regulation of Effector CD8+ T Cells During Mycobacterium Tuberculosis Infection

Regulation of Effector $CD8^+$ T Cells During *Mycobacterium tuberculosis* Infection

A dissertation presented

by

Matthew Gregory Booty

to

The Division of Medical Science

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Immunology

Harvard University

Cambridge, Massachusetts

April, 2015

Dissertation Advisor: Dr. Samuel M. Behar Matthew Gregory Booty

Regulation of Effector $CD8^+$ T Cells During *Mycobacterium tuberculosis* Infection

ABSTRACT

Approximately one-third of the world's population is currently infected with *Mycobacterium tuberculosis* (Mtb), the bacillus that causes tuberculosis. Globally, it is the second leading cause of death by a single infectious agent. An effective vaccine is needed to stop this ongoing pandemic, but efforts to design one are hampered by our limited understanding of host immunity to this pathogen.

$CD8^+$ T cells are elicited during tuberculosis and are required for optimum host resistance. They produce cytokines such as IFN-γ and can directly lyse infected cells. During infection, the expansion and differentiation of effector $CD8^+$ T cells is a dynamically regulated process that is influenced by the inflammatory milieu of the infected host. Currently, the signals governing $CD8^+$ T cell responses during tuberculosis are not well characterized.

Utilizing a mouse model of disease, we address the effects of key cytokines on $CD8^+$ T cells, beginning with IL-12, type 1 interferons (IFN), and IL-27. All three of these cytokines are produced by innate immune cells during tuberculosis and have profound effects on host resistance. IL-12 proves most essential for robust $CD8^+$ T cell expansion and IFN-γ production and also drives the terminal differentiation of short-lived effector cells. However, IL-12 is not acting alone, and type 1 IFN and IL-27 each have non-

redundant roles supporting expansion in infected lungs. Thus, $CD8^+$ T cells reflect the inflammatory environment of the host, responding in different degrees to each cytokine present.

We next examine the role of IL-21, a cytokine produced by activated $CD4^+$ T cells. In the absence of IL-21 signaling, $CD8^+$ T cell expansion and effector functions are severely compromised. IL-21 is also essential to prevent $CD8^+$ T cell exhaustion at later time points during disease. These observations are the first to describe an essential role for IL-21 in the host immune response to Mtb.

Together, these studies establish IL-12 and IL-21 as essential regulators of $CD8^+$ T cells during tuberculosis, and indicate type 1 IFN and IL-27 support expansion in the lungs. We believe these observations have implications for future immunotherapies and rational vaccine design.

Table of Contents

Acknowledgments

For me, the greatest joy of working in science stems from the amazing colleagues that continually surround me. Through my work as a graduate student, many brilliant, dedicated, and charming people supported me, and I acquired a wealth of friends. First, I must express my utmost gratitude and respect for Sam Behar. I can imagine no greater mentor than Sam and my success is a direct result of his guidance. Sam is an exemplary scientists, and his passion, creativity, and critical thinking continue to inspire me.

There are many people to thank in the lab, and I'll start with the original "Behar Kids", Connie Martin, Cláudio Nunes-Alves, and Alyssa Rothchild. They kept long days at work entertaining and were always there to build me up when the experiments wore me down. Together, we formed a family of graduate students and learned from each other along the way. I wouldn't have made it through without them. Now, there is a new group of Behar Kids, Palmira Barreira da Silva, Miye Jacques, Pak Sutiwisesak, and Jason Yang. Their enthusiasm keeps me motivated, and they continue to keep the lab fun and energetic. Through the years, I also received encouragement and advice from two gifted postdocs, Pushpa Jayaraman and Steve Carpenter. I've had the pleasure to call Steve my baymate for the past two years, and he's always been there to share a laugh and keep me sane. Finally, I owe a great debt to the many talented technicians who worked hard to help me succeed, including Danielle Desjardins, Kate Steblenko, and Britni Stowell,

I am thankful for the guidance I received from my Dissertation Advisory Committee: Michael Carroll, Jonanthan Kagan, and Florian Winau and my examination

committee: Michael Brenner, Michael Carroll, Michael Starnbach, and Christopher Sassetti. I want to acknowledge Heinz Remold as well for many enthusiastic and insightful discussions over the years.

Of course, I've received amazing support from my friends and family. In particular, Evan Cale had a remarkably positive impact on my time in Boston. He was the first graduate student I met at Harvard, and we've remained friends since that very day. Finally, I must thank my wonderful parents, Suzanne and Greg, for always encouraging my curiosity and pushing me to excel. They believed in me when I doubted my path and made sure I never gave up.

List of figures

Chapter 2

Chapter 3

Appendix

List of abbreviations

+	having the indicated characteristic (read as “positive”)
-	lacking the indicated characteristic (read as “negative”)
-/-	genetically deficient (read as “knockout”)
+/-	heterozygous
5-LO	5-lipoxygenase
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
APC	antigen presenting cell
β2M	β2-microglobulin
BCG	bacillus Calmette-Guérin
CD	cluster of differentiation
CFU	colony forming units
COX	cyclooxygenase
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DPEC	double positive effector cell
EEC	early effector cell
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescent activated cell sorting (flow cytometry)
GM-CSF	granulocyte macrophage colony-stimulating factor
ICS	intracellular cytokine staining
IFN	interferon(s)
IL	interleukin
iNOS	inducible nitric oxide synthase
KO	genetically deficient (read as “knockout”)
LCMV	lymphocytic choriomeningitis virus
LXA_4	lipoxin A4
MDR-TB	multi-drug resistant tuberculosis
MHC	major histocompatibility complex
MFI	median fluorescent intensity
MPEC	memory precursor effector cell
MSMD	Mendelian susceptibility to mycobacterial diseases
Mtb	*Mycobacterium tuberculosis*
NK	natural killer cell
NKT	natural killer T cell
PAMP	pathogen-associated molecular pattern
PDIM	phthiocerol dimycocerosate
PGE_2	prostaglandin E2
PTGES	prostaglandin E synthase
RD1	region of difference 1
SEM	standard error of the mean
SLEC	short-lived effector cell
SNP	single nucleotide polymorphism

Syt-7	synaptotagmin-7
TAP	transporter associated with antigen processing
TB	tuberculosis
TCR	T cell receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
VSV	vesicular stomatitis virus
VV	vaccinia virus
WHO	world health organization
WT	wild-type
XSCID	X-linked severe combined immunodeficiency
XDR-TB	extensively-drug resistant tuberculosis

Introductory note

The work presented here centers on understanding the regulation of $CD8^+$ T cells during infection with *Mycobacterium tuberculosis* (Mtb). **Chapter 1** contains an overview of the pathogenesis of Mtb and focuses on the events leading up to the priming of the adaptive immune response. The roles of both $CD4^+$ and $CD8^+$ T cells are reviewed and several gaps in our understanding of adaptive immunity during tuberculosis are highlighted. The chapter concludes by raising several key questions regarding the regulation of $CD8^+$ T cells in response to Mtb.

Chapter 2 addresses the impact of IL-12, type 1 interferons (IFN), and IL-27 on the $CD8^+$ T cell response. These cytokines were chosen for two important reasons. First, they differentially regulate susceptibility to tuberculosis. Second, they profoundly influence $CD8^+$ T cell responses in other infections. This chapter begins with a literature review detailing how these cytokines influence host resistance to Mtb and discusses the effects they have on $CD8^+$ T cells in other contexts. The results and implication of our studies in tuberculosis are then presented.

Chapter 3 starts with a review of the biology of IL-21, a relatively new cytokine that is essential for $CD8^+$ T cell responses during chronic viral infections. Currently, the role of IL-21 signaling during tuberculosis is relatively unexplored. Data are presented detailing the effects of IL-21 on the $CD8^+$ T cell response during tuberculosis, and preliminary results are presented that indicate IL-21 is a major mediator of susceptibility. **Chapter 4** briefly summarizes the findings presented in chapters 2 and 3, and discusses some of the implications of this work.

Appendix 1 contains work that indicates cyclooxygenase-2 (COX-2) affects resistance to tuberculosis. Though not directly related to $CD8^+$ T cell biology, the data provide insight into the role of eicosanoids in the host response to Mtb. The appendix begins with a review of our current understanding of these vital signaling molecules and their roles in tuberculosis. The experiments presented indicate that COX-2 is required for host resistance and highlight a role for nonhematopoietic cells in limiting pathology.

Chapter 1:

Introduction

*Portions of the text were adapted from the author's own writing published in:

Behar SM, Carpenter SM, **Booty MG**, Barber DL, Jayaraman P.
Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. Seminars in Immunology 2014;26:559–77.

Nunes-Alves C, **Booty MG**, Carpenter SM, Jayaraman P, Rothchild AC, Behar SM.
In search of a new paradigm for protective immunity to TB. Nature Reviews Microbiology 2014;12:289–99.

Tuberculosis, a continued global health burden

An estimated 9 million new cases of tuberculosis occurred worldwide in 2013 along with 1.5 million deaths (1). Currently, nearly one-third of the world's population is infected with Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis, with roughly 1 in 10 infected individuals developing active disease in their lifetime (2). The majority of infected individuals remain in a state of clinically asymptomatic latent tuberculosis (3), suggesting the immune system is capable of controlling, but not sterilizing the infection. Since 1990, TB mortality rates fell by an estimated 45%, indicating public health efforts are making substantial progress (1); however, the impact of tuberculosis is compounded by the AIDS epidemic and the continued spread of drug-resistant strains of Mtb. In 2013 alone, an estimated 480,000 people developed multi-drug resistant tuberculosis (MDR-TB) and 9% of those patients had extensively drug resistant tuberculosis (XDR-TB) (1). Current treatment regimens take several months to complete, thus an effective vaccine would greatly accelerate the control of tuberculosis and break the cycle of transmission (4). Bacillus Calmette-Guérin (BCG) was introduced as a vaccine almost a century ago, but it's efficacy against pulmonary tuberculosis is highly variable between different populations (5). There are now over a dozen candidate vaccines being tested in clinical trials; however, many preliminary results have been disappointing (4,6-8). There are still large gaps in our understanding of the host's response to tuberculosis, and we lack reliable correlates of protective immunity. Thus, a better understanding of the host's immune response will be essential to designing a successful vaccine.

The intracellular pathogenesis of Mtb

Mtb is a facultative intracellular pathogen that primarily resides in macrophages and other phagocytes, where it is able to survive and replicate despite the hostile conditions within these cells. Mtb enters the host's airways via aerosolized droplets and is subsequently phagocytosed by alveolar macrophages. Phagocytosis of Mtb is triggered through a number of mechanisms, including engagement of pattern recognition receptors (PRRs), C-type lectin receptors, complement receptors (CRs), and Fcγ receptors (FcγRs) (9-11). Recent data suggests Mtb masks its surface pathogen-associated molecular patterns (PAMPs) with lipids, specifically pthiocerol dimycoceroserate (PDIM), in an effort to avoid recruiting highly microbicidal macrophages (12,13).

Once inside the cell, Mtb is capable of blocking phagosome maturation as a means of preventing it's own destruction (14). Phagosome arrest is also postulated to facilitate the evasion of the adaptive immune response by limiting the presentation of antigens on major histocompatibility complex (MHC) class II (15,16). Numerous mycobacterial lipids and proteins are implicated in preventing the acquisition of late endosomal/lysosomal markers as well as blocking the acidification of the phagosome (17). Mycobacteria are thought to replicate within the immature phagosome and are hypothesized to obtain nutrients through interactions with early endosomes (18,19). Stimulation with interferon gamma (IFN-γ) enhances the killing of mycobacteria, in part by promoting the fusion of the mycobacterial phagosome with late endosomes/lysosomes via autophagy (20).

Though Mtb is generally thought to reside and replicate within the phagosome, several groups have observed bacteria in the cytosol (21-26), and it was suggested that Mtb replicates in the cytosol of infected DCs (23). In these systems, escape is only observed with virulent strains and is dependent on the “region of difference 1” (RD1) locus, a major virulence determinant (27). The RD1 locus encodes a novel secretion system, and one of its multiple substrate proteins, ESAT6, can cause membrane damage (28,29). Furthermore, the destabilization of phagosomal membranes likely facilitates the sensing of mycobacterial components by host cytosolic receptors, including nucleotide-binding oligomerization domain (NOD) proteins (10,30). Thus, phagosome destabilization and escape have important implications for the host’s response to Mtb.

Cell death and immunity to Mtb

It is becoming increasingly apparent that cell death is a pivotal axis regulating the outcome of Mtb infections. Virulent strains of Mtb, including H37Rv, primarily induce necrosis in infected macrophages, while H37Ra, an avirulent strain, induces apoptosis (31-33). The direct inhibition of apoptosis contributes to virulence, and Mtb actively blocks apoptosis through multiple mechanisms (34,35). Infection with avirulent bacteria induces the formation of the apoptotic envelope, an insoluble membrane sheath on the cell surface (36), and apoptosis limits the spread of bacteria while enhancing bacterial killing (37-40). Recently, Martin et al. linked apoptosis to bactericidal activity through a mechanism known as efferocytosis (41). In this process, uninfected macrophages engulf apoptotic cells containing Mtb and destroy the bacteria through phagolysosome

fusion. Conversely, necrosis is detrimental to the host, as it facilitates the escape of Mtb from the infected macrophage and promotes the spread of bacteria into new cells. This is corroborated by the identification of the "super-susceptibility to tuberculosis 1" (sst1) locus in mice. Mice carrying the susceptible allele at this locus succumb to infection earlier due to the increased necrosis of macrophages (42).

In addition to a role in innate immunity, the modality of cell death directly influences the adaptive immune response. Apoptosis of infected macrophages can facilitate the priming of $CD8^+$ T cells in a process known as the "Detour Pathway" (43-45). In this pathway, dendritic cells (DCs) phagocytose apoptotic blebs from infected cells and present mycobacterial antigens on CD1 and MHC Class I. The finding that a proapoptotic mutant of Mtb more efficiently primes $CD8^+$ T cells in vivo provides clear support of this model (46). Additionally, manipulation of the host macrophage to preferentially undergo apoptosis accelerates $CD8^+$ T cell priming *in vivo* (47). Based on these observations, the induction of necrosis by virulent Mtb likely delays the adaptive immune response by limiting the efficient presentation of MHC Class I and CD1-restricted antigens.

The initiation of the adaptive immune response

T cell priming is considerably delayed following infection with Mtb, and there is evidence this delay correlates with host susceptibility (48,49). The factors controlling the initiation of the adaptive immune response are incompletely understood, but to some extent, the host influences the process. For example, resistant C57BL/6 mice prime T cells sooner in the lymph node and mount a faster adaptive immune response when

compared to more susceptible C3H mice (48). In mouse studies, aerosol infections are typically done with few bacteria (<200), thus limited amounts of antigen may also influence the generation of the adaptive immune response. However, increasing the inoculum size fails to significantly accelerate T cell priming in the lymph node, suggesting antigen availability is not the limiting factor (50). As mentioned above, Mtb impacts adaptive immunity by inhibiting apoptosis, but it also directly delays priming by inhibiting the maturation of infected DCs (51,52).

Following infection, bacteria must be transferred from the lungs to the draining mediastinal lymph to prime naïve T cells (48,53-56), though priming can occur directly in the lungs under certain circumstances (57). The process of dissemination to the lymph node takes approximately 9-11 days in the mouse model, and a variety of myeloid cells are involved in trafficking the bacteria. $CCR2^{+}$ monocytes are important for delivering Mtb to the lymph node, though these cells still rely on lymph node resident DCs to prime T cells (58). Dendritic cells infected *in vitro* can also carry Mtb to the draining lymph node and, once there, are capable of transferring antigen to uninfected resident DCs (59). Additionally, neutrophils play a role in facilitating dissemination, as the depletion of lung neutrophils further delays the accumulation of Mtb in the lymph node (60). Interestingly, enhanced neutrophil apoptosis accelerates the priming of naïve T cells, suggesting that the trafficking of bacteria may be initiated when antigen presenting cells engulf infected apoptotic neutrophils (61). The transfer of antigen from infected to non-infected cells is commonly observed in studies of priming, likely because directly infected DCs have a diminished ability to prime naïve T cells (52,59). Once bacteria

reach the LN, naïve $CD4^+$ and $CD8^+$ T cells begin to expand and subsequently migrate to the lung to exert their effector functions.

Because priming is delayed following aerosol infection, approximately two weeks pass before effector T cells begin to accumulate in the lungs. This lag is detrimental to the host, as the replication of Mtb is relatively unrestricted during this time. Once activated, T cells halt Mtb growth, and bacterial burden switches from increasing exponentially to a plateau phase (48,54). In resistant strains of mice, this plateau phase is maintained for several months with effector T cells continuing to restrain but not reduce bacteria. The requirement for the adaptive immune response to control the growth of Mtb is best illustrated in RAG knockout (KO) mice, which lack B and T cells. In these mice, Mtb continues to grow exponentially and this plateau phase is never reached (62,63). Shortening the time it takes for effector T cells to reach the lung and inhibit bacterial growth is a major goal of vaccination.

The role of $CD4^+$ T cells

$CD4^+$ T cells are considered the major mediators of protective immunity during tuberculosis, and IFN-γ-producing T_H1 cells are the most prevalent $CD4^+$ subset (64-67). Though far less common, T_H17 cells and regulatory T cells (Tregs) are also present during infection. In particular, T_H17 cells have protective effects during the initial response to Mtb in the lung but become pathogenic if left unchecked (68-71). Overall, the requirement for $CD4^+$ T cells in host protection is most evident in animal models where $CD4^+$ T cells can be selectively depleted. In multiple mouse and primate studies, the loss of $CD4^+$ T cells dramatically increases bacterial burden and worsens disease

(72-76). In humans, HIV induced depletion of $CD4^+$ T cells is one of the greatest risk factors for developing active pulmonary tuberculosis (77).

During infection, $CD4^+$ T cells are a major source of IFN-γ, and data in both mice and humans indicate IFN-γ is essential for protection against Mtb (78-82). Thus, it is assumed $CD4^+$ T cells primarily mediate protection through IFN-γ; however, there is little direct evidence to support this. A recent study demonstrated that $CD4^+$ T cell-derived IFN-γ prolongs survival in infected mice, but even IFN-$\gamma^{-/-}$ $CD4^+$ T confer a level of protection (83). Indeed, several studies indicate effector $CD4^+$ T cells can mediate protection through IFN-γ-independent mechanisms (83-85). Gallegos and colleagues evaluated the cytokines required by $CD4^+$ T cells using transgenic T cells specific for the MHC class II-restricted epitope of the Mtb antigen ESAT6. Surprisingly, antigen-specific $CD4^+$ T cells control bacterial growth even when they lacked IFN-γ, TNF, perforin, or FasL (85). Even cells lacking both IFN-γ and TNF reduced bacterial burden. It is possible that many of these effector functions are redundant for $CD4^+$ T cell-mediated protection, but the data suggest additional effector mechanisms exist to control bacterial growth. Jayaraman et al. recently identified a novel mechanism in which Tim-3, a molecule expressed on the surface of T_H1 cells, directly interacts with macrophages to promote their activation and stimulate bactericidal activity (86). There are likely many effector mechanisms yet to be discovered in T cells.

In addition to their antimicrobial functions, $CD4^+$ T cells play important roles in dampening inflammation and preventing immunopathology. This aspect of the immune response is critical during Mtb infection, and a correlation between host susceptibility and immunopathology has long been observed. Though IFN-γ is one of the major pro-

inflammatory cytokines produced by both $CD4^+$ and $CD8^+$ T cells, it also serves to limit inflammation through multiple distinct mechanisms. IFN-γ inhibits $CD4^+$ T cell production of IL-17, a cytokine that drives inflammation in part by regulating the recruitment of neutrophils (87). Additionally, IFN-γ directly inhibits the accumulation of neutrophils and impairs their survival (84,88). The production of nitric oxide (NO) by the inducible form of nitric oxide synthase (iNOS) is one of the main antimicrobial effectors stimulated by IFN-γ. In addition to controlling bacterial growth, NO inhibits the assembly of the NLRP3 inflammasome and curtails the production of pro-inflammatory IL-1β (89). These examples underscore how a single molecule can have pleiotropic effects in the course of an infection and illustrate how T cells can simultaneously support bactericidal activity while limiting inflammation.

$CD4^+$ T cells are also key sources of other anti-inflammatory meditators. In particular, regulatory T cells (Tregs) are a specially equipped population of cells capable of suppressing inflammation and limiting immune responses. Tregs produce the immunosuppressive cytokines IL-10 and TGF-β and can further suppress immune responses through direct cell-mediated interactions (90). During tuberculosis, Tregs are actually associated with limiting protective immunity and increasing bacterial burden (91-93). However, Tregs can control local tissue damage in other infections (94), and their role in regulating immunopathology during tuberculosis has never been directly addressed.

Both $CD4^+$ and $CD8^+$ T cells are elicited during Mtb infection, and comparatively, $CD4^+$ T cells are considered more protective. Mogues et al. performed the clearest studies supporting this claim by selectively depleting either $CD4^+$ or $CD8^+$ T cells in

thymectomized mice and monitoring survival. Both groups of mice succumb early to aerosol infection, but the loss of $CD4^+$ T cells has a far greater impact on survival (95). Of note, the greatest reduction in survival was observed when both $CD4^+$ and $CD8^+$ T cells were depleted, indicating both cell types are required for optimal resistance. Based on these observations, it appears $CD4^+$ T cells have a dominant role during infection; however, these data should be interpreted with some caution. During viral infections, $CD4^+$ T cells are essential to promote and maintain a robust $CD8^+$ T cell response (96-103). During tuberculosis, it is known that $CD4^+$ T cells enhance $CD8^+$ T cell IFN-γ production and cytolytic activity (83,104,105). Thus, one important $CD4^+$ T cell function is to support the $CD8^+$ T cell response. It is undeniable that $CD4^+$ T cells are essential for resistance to tuberculosis, but we are only beginning to understand all of the mechanisms involved in protection.

The role of $CD8^+$ T cells

Antigen processing

$CD4^+$ T cells typically recognize antigens processed by lysosomal proteolysis in endocytic and phagocytic pathways and presented by professional antigen presenting cells (APCs) (106). In contrast, $CD8^+$ T cells recognize cytosolic antigens that can be processed and presented by almost all cell types. In this process, self and foreign proteins that enter the cytosol are cleaved by the proteasome into short peptides (8-10 amino acids) and transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). Once in the ER, the peptide assembles with

class I MHC heavy chain and β2-microglobulin (β2m) and is transported to the cell surface (107). Exogenous antigens obtained by endocytosis and phagocytosis can enter the MHC class 1 pathway in a process known as cross-presentation. Initially, the impression that $CD4^+$ T cells are more important than $CD8^+$ T cells was reinforced by the differences in class I and class II antigen presentation. Because Mtb was thought to reside exclusively in the phagosome, it seemed logical that the MHC class II pathway would be the dominant form of antigen presentation during infection. Based on our current understanding, we know that $CD8^+$ T cells are primed during tuberculosis and can begin to speculate on the mechanisms involved in the cross-presentation of Mtb antigens.

Secreted Mtb proteins are the primary targets of both $CD4^+$ and $CD8^+$ T cells during tuberculosis (108,109). In particular, ESAT-6 related proteins secreted by specialized type VII secretion systems are prominent $CD8^+$ T cell antigens in both humans and mice (110-119), and their secretion is absolutely required to prime $CD8^+$ T cells (120,121). Following low-dose aerosol infection of C57BL/6 mice, approximately 30-50% of $CD8^+$ T cells in the lungs respond to an epitope in the secreted ESAT-6 related protein TB10.4 (EsxH) (47,117,122). The reasons why CD8+ T cells preferentially recognize secreted antigens are not entirely clear. It is possible that the research is biased simply because secreted antigens are easier to obtain in purified form. However, the immunodominance of many of these antigens is remarkable, and suggests some biological mechanism is at work. Our current understanding of the intracellular pathogenesis of Mtb may offer some clues regarding the prominence of secreted antigens. As previously discussed, there is increasing evidence that Mtb

damages phagosomal membranes and possibly escapes into the cytosol. Destabilizing the phagosome would allow secreted proteins to enter the cytosol where they can be processed for MCH class I presentation. Of course, there are other pathways of cross-presentation, and regardless of the precise mechanism, $CD8^+$ T cells are clearly primed during infection.

$CD8^+$ T cells are required for optimal resistance to tuberculosis

The first data to indicate a protective role for $CD8^+$ T cells came from studies in the mouse model. In 1987, Ian Orme demonstrated that purified $CD4^+$ or $CD8^+$ T cells from the spleens of infected mice conferred protection when transferred into recipients. Specifically, immune $CD8^+$ T cells reduced bacterial burden when transferred into sub-lethally irradiated mice prior to IV infection (123). In the same year, Müller et al. demonstrated that the absence of $CD8^+$ T cells increases susceptibility to Mtb. They observed that the depletion of $CD8^+$ T cells in thymectomized mice reduced their ability to control bacterial growth in the spleen following IV infection (124).

Studies in knockout mice eventually provided the clearest evidence linking $CD8^+$ T cells to resistance. In 1992, Flynn et al. demonstrated that mice lacking β2-microglobulin ($\beta2m^{-/-}$) succumb rapidly to IV infection with Mtb (125). In $\beta2m^{-/-}$ mice, MHC class I does not traffic to the cell surface, and consequently, $CD8^+$ T cells fail to be positively selected during thymic development. Thus, the susceptibility of $\beta2m^{-/-}$ mice was attributed to the absence of $CD8^+$ T cells; however, other antigen presenting molecules, including CD1, also require β2m. Behar and colleagues resolved this by examining the susceptibility of $CD1d^{-/-}$ mice and TAP-$1^{-/-}$ mice (defective in MHC class I

presentation) (126). These experiments demonstrated that antigen presentation by MHC class I was essential for protection, while the loss of CD1d had little impact on host susceptibility in the mouse model. The requirement for $CD8^+$ T cells has since been confirmed using $CD8^{-/-}$ mice and the class I MHC heavy chain knockout (95,127-129). In addition to the use of knockout mice, numerous studies utilizing the adoptive transfer of cells as well as vaccination strategies have demonstrated protective roles for $CD8^+$ T cells (reviewed in (130)).

$CD8^+$ T cells are also critical for immunity in both non-human primate and bovine models of tuberculosis (131-133). In early studies with human samples, $CD8^+$ T cells were identified as a major component of granulomas, and Ag-specific $CD8^+$ T cells were cloned from the pleural fluid of patients with tuberculosis (134,135). Currently, there is no data that directly demonstrates $CD8^+$ T cells are essential for immunity in humans. However, there is considerable evidence that infected people generate Ag-specific $CD8^+$ T cells that express effector functions and are cable of controlling bacterial growth (115,136-140). These observations coupled with studies in animal models indicate that $CD8^+$ T cells are important mediators of immunity to Mtb.

$CD8^+$ T cell effector functions

As previously mentioned, IFN-γ is essential for resistance to tuberculosis (78-82). Though $CD4^+$ T cells are considered a major source of IFN-γ during infection, Mtb-specific $CD8^+$ T cells from mice and humans also produce this important cytokine (110,112,113,141-144). However, it is difficult to demonstrate that IFN-γ from $CD8^+$ T cells is essential for immunity to Mtb. $CD8^+$ T cells require IFN-γ to mediate protection

when transferred into T cell deficient recipients, thus they are capable of supporting IFN-γ-driven protection *in vivo* (145). We recently demonstrated that TB10.4-specific $CD8^+$ T cells require IFN-γ to protect sublethally irradiated mice from aerosol infection (Nunes-Alves et al., *in press*). This protection was dependent on TAP1 expression in the host, indicating that TB10.4-specific $CD8^+$ T cells produced IFN-γ in response to antigen stimulation. Still, IFN-γ-producing $CD8^+$ T cells cannot fully compensate for the protection mediated by $CD4^+$ T cells. $CD8^+$ T cells maintain high levels of IFN-γ in the lungs of $CD4^{-/-}$ mice following IV infection, yet these animals still succumb early to disease (146). In addition to IFN-γ, $CD8^+$ T cells also produce TNF and GM-CSF in response to infection (111,144), but little is known about the role of these cytokines in $CD8^+$ T cell-mediated protection. Though $CD8^+$ T cells clearly make important cytokines, their relative contribution to protective cytokine production remains unclear during the course of natural infection.

One of the most important effector functions of $CD8^+$ T cells is the ability to kill infected cells, and this killing is typically dependent on the TCR. $CD8^+$ T cells that acquire the ability to specifically lyse targets are referred to as cytotoxic T lymphocytes (CTLs). During tuberculosis, CTLs are elicited both in humans and in animal models and have the ability lyse targets *in vitro* and *in vivo* (110,114,147). There are three main pathways that mediate CTL activity: cytotoxic granule exocytosis; Fas/FasL; and TNF secretion (64). $Fas^{-/-}$, $FasL^{-/-}$, and $perforin^{-/-}$ mice are all more susceptible to tuberculosis, suggesting cytolysis is an important mechanism of protection. (127,148).

During cytotoxic granule exocytosis, perforin is essential to allow granzymes to enter the target cell and trigger apoptosis (149), and this molecule is an important

mediator of $CD8^+$ T cell function during tuberculosis. Stimulated $CD8^+$ T cells from the lungs of Mtb-infected mice can lyse infected macrophages *in vitro* in a manner dependent on granule exocytosis (150). *In vivo*, CTL activity can be directly assessed by transferring groups of fluorescently labeled splenocytes into infected animals. These splenocyte populations are pulsed with $CD8^+$ T cell antigens (e.g. TB10.4) or left unpulsed as controls. By comparing the ratio of pulsed to unpulsed splenocytes over time, the specific killing of these cells can be measured. In mice, Ag-specific CTL activity can be detected four weeks after aerosol infection with Mtb and persists for months. Using this system, Woodworth et al. demonstrated that perforin is essential for CTL activity *in vivo* during tuberculosis (151). Furthermore, $perforin^{-/-}$ $CD8^+$ T cells are unable to protect sublethally irradiated mice from aerosol infection. In the absence perforin, residual cytolytic activity is observed and is largely dependent on FasL and TNF signaling. Currently, perforin is the only essential molecule known for $CD8^+$ T cell-mediated cytolysis in the mouse model.

Numerous Mtb-specific human $CD8^+$ T cell lines have cytolytic activity in vitro (115,140,152). Similar to mice, human $CD8^+$ T cells require granule exocytosis to control Mtb growth in infected macrophages, and perforin is also an important effector molecule (153). Additionally, human $CD8^+$ T cells can mediate bacterial control with granulysin, a molecule that has direct antimicrobial effects (153). There is no known murine orthologue of granulysin, suggesting mouse and human $CD8^+$ T cells differ somewhat in terms of their effector functions.

All $CD8^+$ T cell cytolytic pathways result in the target cell undergoing apoptosis, and apoptosis is associated with reduced bacterial burden (154). We hypothesize that

apoptosis leads to bacterial control through efferocytosis, which directly mediates bacterial killings (41). Therefore, $CD8^+$ T cells may promote bacterial control by inducing apoptosis and initiating the process of efferocytosis. This hypothesis remains to be tested, and we are only beginning to understand $CD8^+$ T cell effector functions during tuberculosis.

Unanswered questions

The evidence highlighted in this chapter indicates that $CD8^+$ T cells are important for immunity to Mtb. In particular, animal studies indicate that these cells are required for optimal resistance during tuberculosis. Understanding the role of $CD8^+$ T cells in host immunity will be essential for effective vaccine design, and multiple groups are already targeting $CD8^+$ T cells in vaccine strategies. However, we still know very little about the protective roles of $CD8^+$ T cells and have almost no data on the factors governing $CD8^+$ T cell responses during tuberculosis. In the following chapters, we investigate the regulation of $CD8^+$ T cells, and seek to address several questions:

1. Which cytokine(s) regulate the priming of $CD8^+$ T cells during tuberculosis?
2. Which cytokine(s) expand and maintain $CD8^+$ T cells in the lungs?
3. Which cytokine(s) promote effector functions, including cytokine production and cytolytic activity?
4. Which cytokine(s) regulate $CD8^+$ T cell differentiation and the potential for transition into memory?
5. In what ways do $CD4^+$ T cells support the $CD8^+$ T cell response?

These questions are broad in scope, but we begin by evaluating several key cytokines known to influence $CD8^+$ T cell responses during other infections. We believe that answering these questions will significantly advance our understanding of $CD8^+$ T cells during tuberculosis, and this knowledge has implications for augmenting $CD8^+$ T cell responses during infection, be it by vaccination or other means.

References

1. Global Tuberculosis Report 2014. World Health Organization; 2014.

2. Barry CE, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nature Reviews Microbiology. 2009 Dec;7(12):845–55.

3. Horsburgh CR, Rubin EJ. Clinical practice. Latent tuberculosis infection in the United States. N Engl J Med. 2011 Apr 14;364(15):1441–8.

4. Nunes-Alves C, Booty MG, Carpenter SM, Jayaraman P, Rothchild AC, Behar SM. In search of a new paradigm for protective immunity to TB. Nature Reviews Microbiology. 2014 Apr;12(4):289–99.

5. Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. Lancet. 1995 Nov 18;346(8986):1339–45.

6. Ottenhoff THM, Kaufmann SHE. Vaccines against tuberculosis: where are we and where do we need to go? PLoS Pathog. 2012;8(5):e1002607.

7. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. Lancet. 2013 Mar 23;381(9871):1021–8.

8. Brennan MJ, Stone MR, Evans T. A rational vaccine pipeline for tuberculosis. Int J Tuberc Lung Dis. 2012 Dec;16(12):1566–73.

9. Kleinnijenhuis J, Oosting M, Joosten LAB, Netea MG, van Crevel R. Innate immune recognition of Mycobacterium tuberculosis. Clinical and Developmental Immunology. 2011;2011:405310.

10. Dey B, Bishai WR. Crosstalk between Mycobacterium tuberculosis and the host cell. Seminars in Immunology. 2014 Dec;26(6):486–96.

11. Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev. 2015 Mar;264(1):182–203.

12. Cambier CJ, Takaki KK, Larson RP, Hernandez RE, Tobin DM, Urdahl KB, et al. Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. Nature. 2014 Jan 9;505(7482):218–22.

13. Cambier CJ, Falkow S, Ramakrishnan L. Host evasion and exploitation schemes of Mycobacterium tuberculosis. Cell. 2014 Dec 18;159(7):1497–509.

14. Armstrong JA, Hart PD. Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. J Exp Med. 1971 Sep 1;134(3 Pt 1):713–40.

15. Ramachandra L, Noss E, Boom WH, Harding CV. Processing of Mycobacterium tuberculosis antigen 85B involves intraphagosomal formation of peptide-major histocompatibility complex II complexes and is inhibited by live bacilli that decrease phagosome maturation. J Exp Med. 2001 Nov 19;194(10):1421–32.

16. Singh CR, Moulton RA, Armitige LY, Bidani A, Snuggs M, Dhandayuthapani S, et al. Processing and presentation of a mycobacterial antigen 85B epitope by murine macrophages is dependent on the phagosomal acquisition of vacuolar proton ATPase and in situ activation of cathepsin D. J Immunol. 2006 Sep 1;177(5):3250–9.

17. Philips JA. Mycobacterial manipulation of vacuolar sorting. Cell Microbiol. 2008 Dec;10(12):2408–15.

18. Clemens DL, Horwitz MA. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. J Exp Med. 1995 Jan 1;181(1):257–70.

19. Sturgill-Koszycki S, Schaible UE, Russell DG. Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. The EMBO Journal. 1996 Dec 16;15(24):6960–8.

20. Singh SB. Human IRGM Induces Autophagy to Eliminate Intracellular Mycobacteria. Science. 2006 Sep 8;313(5792):1438–41.

21. Myrvik QN, Leake ES, Wright MJ. Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of Mycobacterium tuberculosis. A correlate of virulence. Am Rev Respir Dis. 1984 Feb;129(2):322–8.

22. McDonough KA, Kress Y, Bloom BR. The interaction of Mycobacterium tuberculosis with macrophages: a study of phagolysosome fusion. Infect Agents Dis. 1993 Aug;2(4):232–5.

23. van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, et al. M. tuberculosis and M. leprae Translocate from the Phagolysosome to the Cytosol in Myeloid Cells. Cell. 2007 Jun;129(7):1287–98.

24. Simeone R, Bobard A, Lippmann J, Bitter W, Majlessi L, Brosch R, et al. Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. Simeone R, Bobard A, Lippmann J, Bitter W, Majlessi L, Brosch R, et al., editors. PLoS Pathog. 2012 Feb;8(2):e1002507.

25. Hoshino A, Hanada S, Yamada H, Mii S, Takahashi M, Mitarai S, et al. Mycobacterium tuberculosis escapes from the phagosomes of infected human osteoclasts

reprograms osteoclast development via dysregulation of cytokines and chemokines. Pathog Dis. 2014 Feb;70(1):28–39.

26. Rahman A, Sobia P, Gupta N, Kaer LV, Das G. Mycobacterium tuberculosis subverts the TLR-2-MyD88 pathway to facilitate its translocation into the cytosol. PLoS ONE. 2014;9(1):e86886.

27. Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, et al. The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc Natl Acad Sci USA. 2003 Oct 14;100(21):12420–5.

28. de Jonge MI, Pehau-Arnaudet G, Fretz MM, Romain F, Bottai D, Brodin P, et al. ESAT-6 from Mycobacterium tuberculosis Dissociates from Its Putative Chaperone CFP-10 under Acidic Conditions and Exhibits Membrane-Lysing Activity. Journal of Bacteriology. 2007 Aug 1;189(16):6028–34.

29. Smith J, Manoranjan J, Pan M, Bohsali A, Xu J, Liu J, et al. Evidence for Pore Formation in Host Cell Membranes by ESX-1-Secreted ESAT-6 and Its Role in Mycobacterium marinum Escape from the Vacuole. Infection and Immunity. 2008 Nov 17;76(12):5478–87.

30. Pandey AK, Yang Y, Jiang Z, Fortune SM, Coulombe F, Behr MA, et al. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to Mycobacterium tuberculosis. PLoS Pathog. 2009 Jul;5(7):e1000500.

31. Park JS. Virulent clinical isolates of Mycobacterium tuberculosis grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. Journal of Leukocyte Biology. 2005 Oct 4;79(1):80–6.

32. Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, et al. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. Infection and Immunity. 1997 Jan;65(1):298–304.

33. Chen M, Gan H, Remold HG. A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. J Immunol. 2006 Mar 15;176(6):3707–16.

34. Behar SM, Martin CJ, Booty MG, Nishimura T, Zhao X, Gan H-X, et al. Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. Mucosal Immunology. Nature Publishing Group; 2011 Feb 9;:1–9.

35. Velmurugan K, Chen B, Miller JL, Azogue S, Gurses S, Hsu T, et al. Mycobacterium tuberculosis nuoG is a virulence gene that inhibits apoptosis of infected host cells. PLoS Pathog. 2007 Jul;3(7):e110.

36. Gan H, Lee J, Ren F, Chen M, Kornfeld H, Remold HG. Mycobacterium tuberculosis blocks crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages to maintain virulence. Nat Immunol. 2008 Sep 14;9(10):1189–97.

37. Fratazzi C, Arbeit RD, Carini C, Remold HG. Programmed cell death of Mycobacterium avium serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. J Immunol. 1997 May 1;158(9):4320–7.

38. Sly LM, Hingley-Wilson SM, Reiner NE, McMaster WR. Survival of Mycobacterium tuberculosis in host macrophages involves resistance to apoptosis dependent upon induction of antiapoptotic Bcl-2 family member Mcl-1. J Immunol. 2003 Jan 1;170(1):430–7.

39. Molloy A, Laochumroonvorapong P, Kaplan G. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. J Exp Med. Rockefeller Univ Press; 1994;180(4):1499–509.

40. Oddo M, Renno T, Attinger A, Bakker T, MACDONALD HR, Meylan PR. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. J Immunol. 1998 Jun 1;160(11):5448–54.

41. Martin CJ, Booty MG, Rosebrock TR, Nunes-Alves C, Desjardins DM, Keren I, et al. Efferocytosis is an innate antibacterial mechanism. Cell Host Microbe. 2012 Sep 13;12(3):289–300.

42. Pan H, Yan B-S, Rojas M, Shebzukhov YV, Zhou H, Kobzik L, et al. Ipr1 gene mediates innate immunity to tuberculosis. Nature. 2005 Apr 7;434(7034):767–72.

43. Schaible UE, Winau F, Sieling PA, Fischer K, Collins HL, Hagens K, et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. Nature Medicine. 2003 Aug;9(8):1039–46.

44. Winau F, Weber S, Sad S, de Diego J, Hoops SL, Breiden B, et al. Apoptotic Vesicles Crossprime CD8 T Cells and Protect against Tuberculosis. Immunity. 2006 Jan;24(1):105–17.

45. Winau F, Kaufmann SHE, Schaible UE. Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria. Cell Microbiol. 2004 Jun 4;6(7):599–607.

46. Hinchey J, Lee S, Jeon BY, Basaraba RJ, Venkataswamy MM, Chen B, et al. Enhanced priming of adaptive immunity by a proapoptotic mutant of Mycobacterium tuberculosis. J Clin Invest. 2007 Aug 1;117(8):2279–88.

47. Divangahi M, Desjardins D, Nunes-Alves C, Remold HG, Behar SM. Eicosanoid pathways regulate adaptive immunity to Mycobacterium tuberculosis. Nat

Immunol. 2010 Aug;11(8):751–8.

48. Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. Infection and Immunity. 2002 Aug;70(8):4501–9.

49. Tian T, Woodworth J, Sköld M, Behar SM. In vivo depletion of CD11c+ cells delays the CD4+ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection. J Immunol. Am Assoc Immnol; 2005;175(5):3268–72.

50. Myers AJ, Marino S, Kirschner DE, Flynn JL. Inoculation dose of Mycobacterium tuberculosis does not influence priming of T cell responses in lymph nodes. The Journal of Immunology. 2013 May 1;190(9):4707–16.

51. Hanekom WA, Mendillo M, Manca C, Haslett PAJ, Siddiqui MR, Barry C, et al. Mycobacterium tuberculosis inhibits maturation of human monocyte-derived dendritic cells in vitro. J Infect Dis. 2003 Jul 15;188(2):257–66.

52. Wolf AJ, Linas B, Trevejo-Nuñez GJ, Kincaid E, Tamura T, Takatsu K, et al. Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. J Immunol. 2007 Aug 15;179(4):2509–19.

53. Reiley WW, Shafiani S, Wittmer ST, Tucker-Heard G, Moon JJ, Jenkins MK, et al. Distinct functions of antigen-specific CD4 T cells during murine Mycobacterium tuberculosis infection. Proceedings of the National Academy of Sciences. 2010 Nov 9;107(45):19408–13.

54. Wolf AJ, Desvignes L, Linas B, Banaiee N, Tamura T, Takatsu K, et al. Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. Journal of Experimental Medicine. 2008 Jan 21;205(1):105–15.

55. Reiley WW, Calayag MD, Wittmer ST, Huntington JL, Pearl JE, Fountain JJ, et al. ESAT-6-specific CD4 T cell responses to aerosol Mycobacterium tuberculosis infection are initiated in the mediastinal lymph nodes. Proceedings of the National Academy of Sciences. 2008 Aug 5;105(31):10961–6.

56. Gallegos AM, Pamer EG, Glickman MS. Delayed protection by ESAT-6-specific effector CD4+ T cells after airborne M. tuberculosis infection. Journal of Experimental Medicine. 2008 Sep 29;205(10):2359–68.

57. Olmos S, Stukes S, Ernst JD. Ectopic activation of Mycobacterium tuberculosis-specific CD4+ T cells in lungs of CCR7−/− mice. J Immunol. Am Assoc Immnol; 2010;184(2):895–901.

58. Samstein M, Schreiber HA, Leiner IM, Susac B, Glickman MS, Pamer EG. Essential yet limited role for CCR2+ inflammatory monocytes during Mycobacterium

tuberculosis-specific T cell priming. Elife. 2013;2:e01086.

59. Srivastava S, Ernst JD. Cell-to-Cell Transfer of M. tuberculosis Antigens Optimizes CD4 T Cell Priming. Cell Host Microbe. 2014 Jun 11;15(6):741–52.

60. Blomgran R, Ernst JD. Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection. The Journal of Immunology. 2011 Jun 15;186(12):7110–9.

61. Blomgran R, Desvignes L, Briken V, Ernst JD. Mycobacterium tuberculosis inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. Cell Host Microbe. 2012 Jan 19;11(1):81–90.

62. Ehlers S, Benini J, Held HD, Roeck C, Alber G, Uhlig S. Alphabeta T cell receptor-positive cells and interferon-gamma, but not inducible nitric oxide synthase, are critical for granuloma necrosis in a mouse model of mycobacteria-induced pulmonary immunopathology. J Exp Med. 2001 Dec 17;194(12):1847–59.

63. Feng CG, Jankovic D, Kullberg M, Cheever A, Scanga CA, Hieny S, et al. Maintenance of pulmonary Th1 effector function in chronic tuberculosis requires persistent IL-12 production. J Immunol. 2005 Apr 1;174(7):4185–92.

64. Behar SM, Carpenter SM, Booty MG, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. Seminars in Immunology. 2014 Dec;26(6):559–77.

65. North RJ, Jung Y-J. Immunity to Tuberculosis. Annu Rev Immunol. 2004 Apr;22(1):599–623.

66. Cooper AM. Cell-Mediated Immune Responses in Tuberculosis. Annu Rev Immunol. 2009 Apr;27(1):393–422.

67. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MPR. The immune response in tuberculosis. Annu Rev Immunol. 2013;31:475–527.

68. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol. 2007 Apr;8(4):369–77.

69. Khader SA, Guglani L, Rangel Moreno J, Gopal R, Junecko BAF, Fountain JJ, et al. IL-23 is required for long-term control of Mycobacterium tuberculosis and B cell follicle formation in the infected lung. The Journal of Immunology. 2011 Nov 15;187(10):5402–7.

70. Wozniak TM, Saunders BM, Ryan AA, Britton WJ. Mycobacterium bovis BCG-specific Th17 cells confer partial protection against Mycobacterium tuberculosis infection

in the absence of gamma interferon. Infection and Immunity. 2010 Oct;78(10):4187–94.

71. Cruz A, Fraga AG, Fountain JJ, Rangel Moreno J, Torrado E, Saraiva M, et al. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. Journal of Experimental Medicine. 2010 Aug 2;207(8):1609–16.

72. Flory CM, Hubbard RD, Collins FM. Effects of in vivo T lymphocyte subset depletion on mycobacterial infections in mice. Journal of Leukocyte Biology. 1992 Mar;51(3):225–9.

73. Pedrazzini T, Hug K, Louis JA. Importance of L3T4+ and Lyt-2+ cells in the immunologic control of infection with Mycobacterium bovis strain bacillus Calmette-Guérin in mice. Assessment by elimination of T cell subsets in vivo. J Immunol. 1987 Sep 15;139(6):2032–7.

74. Scanga CA, Mohan VP, Yu K, Joseph H, Tanaka K, Chan J, et al. Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. J Exp Med. 2000 Aug 7;192(3):347–58.

75. Diedrich CR, Mattila JT, Klein E, Janssen C, Phuah J, Sturgeon TJ, et al. Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T cell depletion and not virus load. PLoS ONE. 2010;5(3):e9611.

76. Lin PL, Rutledge T, Green AM, Bigbee M, Fuhrman C, Klein E, et al. CD4 T cell depletion exacerbates acute Mycobacterium tuberculosis while reactivation of latent infection is dependent on severity of tissue depletion in cynomolgus macaques. AIDS Res Hum Retroviruses. 2012 Dec;28(12):1693–702.

77. Geldmacher C, Zumla A, Hoelscher M. Interaction between HIV and Mycobacterium tuberculosis: HIV-1-induced CD4 T-cell depletion and the development of active tuberculosis. Curr Opin HIV AIDS. 2012 May;7(3):268–75.

78. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science. 1993 Mar 19;259(5102):1739–42.

79. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med. 1993 Dec 1;178(6):2249–54.

80. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med. 1993 Dec 1;178(6):2243–7.

81. Newport MJ, Huxley CM, Huston S, Hawrylowicz CM, Oostra BA, Williamson R, et al. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N Engl J Med. 1996 Dec 26;335(26):1941–9.

82. Bustamante J, Boisson-Dupuis S, Abel L, Casanova J-L. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-γ immunity. Seminars in Immunology. 2014 Dec;26(6):454–70.

83. Green AM, Difazio R, Flynn JL. IFN-γ from CD4 T Cells Is Essential for Host Survival and Enhances CD8 T Cell Function during Mycobacterium tuberculosis Infection. The Journal of Immunology. 2013 Jan 1;190(1):270–7.

84. Nandi B, Behar SM. Regulation of neutrophils by interferon- limits lung inflammation during tuberculosis infection. Journal of Experimental Medicine. 2011 Oct 24;208(11):2251–62.

85. Gallegos AM, van Heijst JWJ, Samstein M, Su X, Pamer EG, Glickman MS. A gamma interferon independent mechanism of CD4 T cell mediated control of M. tuberculosis infection in vivo. PLoS Pathog. 2011 May;7(5):e1002052.

86. Jayaraman P, Sada-Ovalle I, Beladi S, Anderson AC, Dardalhon V, Hotta C, et al. Tim3 binding to galectin-9 stimulates antimicrobial immunity. Journal of Experimental Medicine. 2010 Oct 25;207(11):2343–54.

87. Cruz A, Khader SA, Torrado E, Fraga A, Pearl JE, Pedrosa J, et al. Cutting edge: IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. J Immunol. 2006 Aug 1;177(3):1416–20.

88. Desvignes L, Ernst JD. Interferon-gamma-responsive nonhematopoietic cells regulate the immune response to Mycobacterium tuberculosis. Immunity. 2009 Dec 18;31(6):974–85.

89. Mishra BB, Rathinam VAK, Martens GW, Martinot AJ, Kornfeld H, Fitzgerald KA, et al. Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1β. Nat Immunol. 2013 Jan;14(1):52–60.

90. Josefowicz SZ, Lu L-F, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol. 2012;30:531–64.

91. Kursar M, Koch M, Mittrücker H-W, Nouailles G, Bonhagen K, Kamradt T, et al. Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. J Immunol. 2007 Mar 1;178(5):2661–5.

92. Scott-Browne JP, Shafiani S, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, Rudensky AY, et al. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. J Exp Med. 2007 Sep 3;204(9):2159–69.

93. Shafiani S, Tucker-Heard G, Kariyone A, Takatsu K, Urdahl KB. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. Journal of Experimental Medicine. 2010 Jul 5;207(7):1409–20.

94. Sehrawat S, Rouse BT. Tregs and infections: on the potential value of modifying their function. Journal of Leukocyte Biology. 2011 Dec;90(6):1079–87.

95. Mogues T, Goodrich ME, Ryan L, LaCourse R, North RJ. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. J Exp Med. 2001 Feb 5;193(3):271–80.

96. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. Journal of Virology. 1994 Dec;68(12):8056–63.

97. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med. 1998 Dec 21;188(12):2205–13.

98. Fuller MJ, Khanolkar A, Tebo AE, Zajac AJ. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. J Immunol. 2004 Apr 1;172(7):4204–14.

99. Battegay M, Moskophidis D, Rahemtulla A, Hengartner H, Mak TW, Zinkernagel RM. Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. Journal of Virology. 1994 Jul;68(7):4700–4.

100. Sun JC, Williams MA, Bevan MJ. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. Nat Immunol. 2004 Sep;5(9):927–33.

101. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. Science. 2009 Jun 19;324(5934):1572–6.

102. Fröhlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. Science. 2009 Jun 19;324(5934):1576–80.

103. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. Science. 2009 Jun 19;324(5934):1569–72.

104. Serbina NV, Lazarevic V, Flynn JL. CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during Mycobacterium tuberculosis infection. J Immunol. 2001 Dec 15;167(12):6991–7000.

105. Bold TD, Ernst JD. CD4+ T Cell-Dependent IFN- Production by CD8+ Effector T Cells in Mycobacterium tuberculosis Infection. The Journal of Immunology. 2012 Aug

20;189(5):2530–6.

106. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. Annu Rev Immunol. 2013;31:443–73.

107. Neefjes J, Jongsma MLM, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nature Reviews Immunology. 2011 Dec;11(12):823–36.

108. Dillon DC, Alderson MR, Day CH, Bement T, Campos-Neto A, Skeiky YA, et al. Molecular and immunological characterization of Mycobacterium tuberculosis CFP-10, an immunodiagnostic antigen missing in Mycobacterium bovis BCG. J Clin Microbiol. 2000 Sep;38(9):3285–90.

109. Behar SM. Antigen-specific CD8(+) T cells and protective immunity to tuberculosis. Adv Exp Med Biol. 2013;783:141–63.

110. Kamath AB, Woodworth J, Xiong X, Taylor C, Weng Y, Behar SM. Cytolytic CD8+ T cells recognizing CFP10 are recruited to the lung after Mycobacterium tuberculosis infection. J Exp Med. 2004 Dec 6;200(11):1479–89.

111. Kamath A, Woodworth JSM, Behar SM. Antigen-specific CD8+ T cells and the development of central memory during Mycobacterium tuberculosis infection. J Immunol. 2006 Nov 1;177(9):6361–9.

112. Lewinsohn DM, Zhu L, Madison VJ, Dillon DC, Fling SP, Reed SG, et al. Classically restricted human CD8+ T lymphocytes derived from Mycobacterium tuberculosis-infected cells: definition of antigenic specificity. J Immunol. 2001 Jan 1;166(1):439–46.

113. Majlessi L, Rojas M-J, Brodin P, Leclerc C. CD8+-T-cell responses of Mycobacterium-infected mice to a newly identified major histocompatibility complex class I-restricted epitope shared by proteins of the ESAT-6 family. Infection and Immunity. 2003 Dec;71(12):7173–7.

114. Pathan AA, Wilkinson KA, Wilkinson RJ, Latif M, McShane H, Pasvol G, et al. High frequencies of circulating IFN-gamma-secreting CD8 cytotoxic T cells specific for a novel MHC class I-restricted Mycobacterium tuberculosis epitope in M. tuberculosis-infected subjects without disease. Eur J Immunol. 2000 Sep;30(9):2713–21.

115. Lalvani A, Brookes R, Wilkinson RJ, Malin AS, Pathan AA, Andersen P, et al. Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for Mycobacterium tuberculosis. Proc Natl Acad Sci USA. 1998 Jan 6;95(1):270–5.

116. Brodin P, Rosenkrands I, Andersen P, Cole ST, Brosch R. ESAT-6 proteins: protective antigens and virulence factors? Trends in Microbiology. 2004

Nov;12(11):500–8.

117. Billeskov R, Vingsbo-Lundberg C, Andersen P, Dietrich J. Induction of CD8 T cells against a novel epitope in TB10.4: correlation with mycobacterial virulence and the presence of a functional region of difference-1. J Immunol. 2007 Sep 15;179(6):3973–81.

118. Billeskov R, Grandal MV, Poulsen C, Christensen JP, Winther N, Vingsbo-Lundberg C, et al. Difference in TB10.4 T-cell epitope recognition following immunization with recombinant TB10.4, BCG or infection with Mycobacterium tuberculosis. Eur J Immunol. 2010 May;40(5):1342–54.

119. Skjøt RLV, Brock I, Arend SM, Munk ME, Theisen M, Ottenhoff THM, et al. Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. Infection and Immunity. 2002 Oct;70(10):5446–53.

120. Woodworth JS, Fortune SM, Behar SM. Bacterial protein secretion is required for priming of CD8+ T cells specific for the Mycobacterium tuberculosis antigen CFP10. Infection and Immunity. 2008 Sep;76(9):4199–205.

121. Garces A, Atmakuri K, Chase MR, Woodworth JS, Krastins B, Rothchild AC, et al. EspA acts as a critical mediator of ESX1-dependent virulence in Mycobacterium tuberculosis by affecting bacterial cell wall integrity. PLoS Pathog. 2010;6(6):e1000957.

122. Nobrega C, Nunes-Alves C, Cerqueira-Rodrigues B, Roque S, Barreira-Silva P, Behar SM, et al. T cells home to the thymus and control infection. The Journal of Immunology. 2013 Feb 15;190(4):1646–58.

123. Orme IM. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with Mycobacterium tuberculosis. J Immunol. 1987 Jan 1;138(1):293–8.

124. Müller I, Cobbold SP, Waldmann H, Kaufmann SH. Impaired resistance to Mycobacterium tuberculosis infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. Infection and Immunity. 1987 Sep;55(9):2037–41.

125. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. Proc Natl Acad Sci USA. 1992 Dec 15;89(24):12013–7.

126. Behar SM, Dascher CC, Grusby MJ, Wang CR, Brenner MB. Susceptibility of mice deficient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. J Exp Med. 1999 Jun 21;189(12):1973–80.

127. Sousa AO, Mazzaccaro RJ, Russell RG, Lee FK, Turner OC, Hong S, et al. Relative

contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. Proc Natl Acad Sci USA. 2000 Apr 11;97(8):4204–8.

128. Urdahl KB, Liggitt D, Bevan MJ. CD8+ T cells accumulate in the lungs of Mycobacterium tuberculosis-infected Kb-/-Db-/- mice, but provide minimal protection. J Immunol. 2003 Feb 15;170(4):1987–94.

129. Rolph MS, Raupach B, Köbernick HH, Collins HL, Pérarnau B, Lemonnier FA, et al. MHC class Ia-restricted T cells partially account for beta2-microglobulin-dependent resistance to Mycobacterium tuberculosis. Eur J Immunol. 2001 Jun;31(6):1944–9.

130. Woodworth JSM, Behar SM. Mycobacterium tuberculosis-specific CD8+ T cells and their role in immunity. Crit Rev Immunol. 2006;26(4):317–52.

131. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, et al. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. PLoS Pathog. 2009 Apr;5(4):e1000392.

132. Villarreal-Ramos B, McAulay M, Chance V, Martin M, Morgan J, Howard CJ. Investigation of the role of CD8+ T cells in bovine tuberculosis in vivo. Infection and Immunity. 2003 Aug;71(8):4297–303.

133. Palmer MV, Waters WR, Thacker TC. Lesion development and immunohistochemical changes in granulomas from cattle experimentally infected with Mycobacterium bovis. Vet Pathol. 2007 Nov;44(6):863–74.

134. Randhawa PS. Lymphocyte subsets in granulomas of human tuberculosis: an in situ immunofluorescence study using monoclonal antibodies. Pathology. 1990 Jul;22(3):153–5.

135. Rees A, Scoging A, Mehlert A, Young DB, Ivanyi J. Specificity of proliferative response of human CD8 clones to mycobacterial antigens. Eur J Immunol. 1988 Dec;18(12):1881–7.

136. Lewinsohn DM, Alderson MR, Briden AL, Riddell SR, Reed SG, Grabstein KH. Characterization of human CD8+ T cells reactive with Mycobacterium tuberculosis-infected antigen-presenting cells. J Exp Med. 1998 May 18;187(10):1633–40.

137. Lewinsohn DM, Briden AL, Reed SG, Grabstein KH, Alderson MR. Mycobacterium tuberculosis-reactive CD8+ T lymphocytes: the relative contribution of classical versus nonclassical HLA restriction. J Immunol. 2000 Jul 15;165(2):925–30.

138. Lewinsohn DA, Winata E, Swarbrick GM, Tanner KE, Cook MS, Null MD, et al. Immunodominant tuberculosis CD8 antigens preferentially restricted by HLA-B. PLoS Pathog. 2007 Sep 21;3(9):1240–9.

139. Canaday DH, Ziebold C, Noss EH, Chervenak KA, Harding CV, Boom WH. Activation

of human CD8+ alpha beta TCR+ cells by Mycobacterium tuberculosis via an alternate class I MHC antigen-processing pathway. J Immunol. 1999 Jan 1;162(1):372–9.

140. Mohagheghpour N, Gammon D, Kawamura LM, van Vollenhoven A, Benike CJ, Engleman EG. CTL response to Mycobacterium tuberculosis: identification of an immunogenic epitope in the 19-kDa lipoprotein. J Immunol. 1998 Sep 1;161(5):2400–6.

141. Shams H, Wizel B, Weis SE, Samten B, Barnes PF. Contribution of CD8(+) T cells to gamma interferon production in human tuberculosis. Infection and Immunity. 2001 May;69(5):3497–501.

142. Feng CG, Bean AG, Hooi H, Briscoe H, Britton WJ. Increase in gamma interferon-secreting CD8(+), as well as CD4(+), T cells in lungs following aerosol infection with Mycobacterium tuberculosis. Infection and Immunity. 1999 Jul;67(7):3242–7.

143. Serbina NV, Flynn JL. Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of Mycobacterium tuberculosis-infected mice. Infection and Immunity. 1999 Aug;67(8):3980–8.

144. Tully G, Kortsik C, Höhn H, Zehbe I, Hitzler WE, Neukirch C, et al. Highly focused T cell responses in latent human pulmonary Mycobacterium tuberculosis infection. J Immunol. 2005 Feb 15;174(4):2174–84.

145. Tascon RE, Stavropoulos E, Lukacs KV, Colston MJ. Protection against Mycobacterium tuberculosis infection by CD8+ T cells requires the production of gamma interferon. Infection and Immunity. 1998 Feb;66(2):830–4.

146. Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. J Immunol. 1999 May 1;162(9):5407–16.

147. Skinner MA, Parlane N, McCarthy A, Buddle BM. Cytotoxic T-cell responses to Mycobacterium bovis during experimental infection of cattle with bovine tuberculosis. Immunology. 2003 Oct;110(2):234–41.

148. Turner J, D'Souza CD, Pearl JE, Marietta P, Noel M, Frank AA, et al. CD8- and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. American Journal of Respiratory Cell and Molecular Biology. 2001 Feb;24(2):203–9.

149. de Saint Basile G, Ménasché G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. Nature Reviews Immunology. 2010 Aug;10(8):568–79.

150. Serbina NV, Liu CC, Scanga CA, Flynn JL. CD8+ CTL from lungs of Mycobacterium

tuberculosis-infected mice express perforin in vivo and lyse infected macrophages. J Immunol. 2000 Jul 1;165(1):353–63.

151. Woodworth JS, Wu Y, Behar SM. Mycobacterium tuberculosis-specific CD8+ T cells require perforin to kill target cells and provide protection in vivo. The Journal of Immunology. 2008 Dec 15;181(12):8595–603.

152. De Libero G, Flesch I, Kaufmann SH. Mycobacteria-reactive Lyt-2+ T cell lines. Eur J Immunol. 1988 Jan;18(1):59–66.

153. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science. 1998 Oct 2;282(5386):121–5.

154. Behar SM, Divangahi M, Remold HG. Evasion of innate immunity by Mycobacterium tuberculosis: is death an exit strategy? Nature Reviews Microbiology. Nature Publishing Group; 2010 Aug 2;8(9):668–74.

Chapter 2:

IL-12, type 1 IFN, and IL-27 differentially regulate $CD8^+$ T cell expansion and function during tuberculosis

This work is adapted from an unpublished manuscript pending submission. The work presented included assistance from the following individuals: Cláudio Nunes-Alves, Stephen M. Carpenter, and Pushpa Jayaraman (University of Massachusetts Medical School, Worcester, MA).

*Portions of the text were adapted from the author's own writing published in:

Behar SM, Carpenter SM, **Booty MG**, Barber DL, Jayaraman P.
Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. Seminars in Immunology 2014;26:559–77.

Abstract

The differentiation of effector and memory $CD8^+$ T cells is a dynamically regulated process that varies considerably during different infections and is influenced by the inflammatory milieu of the host. Here, we define three signals regulating $CD8^+$ T cell responses during tuberculosis by focusing on cytokines known to impact disease outcome: IL-12, type 1 interferons (IFN), and IL-27.

IL-12, type 1 IFN, and IL-27 are all elicited during tuberculosis and have profound effects on host resistance. IL-12 is required for resistance in mice and humans, and has an essential role in promoting $CD4^+$ T cell expansion and IFN-γ production. In contrast, IL-27 acts as an immunoregulatory cytokine and can dampen $CD4^+$ T cell responses. Type 1 IFN has multiple effects during infection, but its overproduction is detrimental to the host. The increased resistance of $IFNAR^{-/-}$ mice underscores this fact. In other infections, all three of these cytokines can profoundly affect $CD8^+$ T cell expansion and function; however, their effect on $CD8^+$ T cells during tuberculosis is largely unknown.

Using mixed bone marrow chimeras, we compared wild type and receptor knockout $CD8^+$ T cells within the same mouse following aerosol infection with *M. tuberculosis* (Mtb). Four weeks post-infection, all three cytokines were required for efficient $CD8^+$ T cell expansion in the lungs, with IL-12 showing the greatest contribution. We next determined if these cytokines directly promote priming or are required only for expansion in the lungs. Utilizing retrogenic $CD8^+$ T cells that express a T cell receptor specific for the Mtb antigen TB10.4, we observed that IL-12 is the dominant cytokine driving $CD8^+$ T cell priming in the lymph node and expansion in the

lungs. Type 1 interferons and IL-27 appear to have non-redundant roles in supporting pulmonary $CD8^+$ T cell expansion.

In addition to expansion, these cytokines regulate the differentiation and function of $CD8^+$ T cells during tuberculosis. All three cytokines were necessary for the induction of granzyme B; however, cells still retained a high degree of cytolytic ability. IL-12 was the only cytokine necessary for the differentiation of short-lived effector cells (SLECs) and IFN-γ production. These data demonstrate distinct and overlapping roles for each of the cytokines examined and underscore the complexities of $CD8^+$ T cell regulation during tuberculosis.

Introduction

In this chapter, we examine the regulation of $CD8^+$ T cells during tuberculosis by focusing on three key cytokines: IL-12, type 1 IFN, and IL-27. There are two main reasons why we chose these particular cytokines. First, all three dramatically influence susceptibility to tuberculosis. This chapter begins with a review of these cytokines and explains their impact on immunity to Mtb. The second reason for examining these cytokines is that they are profoundly important for regulating $CD8^+$ T cells during other infections. In particular, IL-12 and type 1 IFN are known as "Signal 3" cytokines, because they provide a third signal necessary for $CD8^+$ T cell priming in many systems. The introduction ends with a review of signal 3 cytokines and their roles in other infections.

The IL-12 family of cytokines

Interleukin 12 (IL-12) was first identified in the supernatant of Epstein-Bar virus (EBV)-transformed B cell lines and was shown to enhance IFN-γ production, cytotoxicity, and proliferation in NK cells (1,2). Following this discovery, IL-12 production by innate immune cells, including macrophages and dendritic cells (DCs), was revealed to drive the T_H1 polarization of naïve $CD4^+$ T cells (3-8). The hallmark of T_H1 polarization is the production of IFN-γ, making IL-12 an important regulator of immune responses to intracellular bacteria and protozoa. Intriguingly, the avirulent *M. tuberculosis* (Mtb) strain H37Ra was one of the earliest stimulants shown to induce IL-12 in peripheral blood mononuclear cells (PBMCs) (6).

Historically, IL-12 is the first cytokine to connect innate and adaptive immunity, and it's discovery supports a central paradigm in which innate cells direct the development of adaptive immune responses. Years later, IL-23 and IL-27 were identified based on homology searches, becoming members of what is now the IL-12 family of cytokines (9,10). Like IL-12, macrophages and DCs can produce IL-23 and IL-27 in response to infection, and these cytokines influence T cell function. IL-35 is the newest member of this family and is unique in that it is a potent inhibitory cytokine primarily secreted by regulatory T cells (Tregs) (11).

The IL-12 family is the only heterodimeric cytokine family, and chain sharing is a common attribute of both the cytokines and their receptors (12,13). IL-12 and IL-23 are disulfide-linked heterodimers consisting of p35/p40 and p19/p40, respectively. IL-27 and IL-35 lack the disulfide linkage and are comprised of p28/Ebi3 and p35/Ebi3, respectively. Interpreting the various functions of IL-12 family members can prove challenging, because multiple cytokines share the same chains (e.g. IL-12 and IL-23 share the same β-chain, p40). This added complexity continues with the cytokine receptors, which also consist of two chains. IL-12 signals through IL-12Rβ1 and IL-12Rβ2, and IL-23 uses IL-12Rβ1 and IL-23R. IL-27 interacts with IL-27Rα (WSX-1) and gp130, and IL-35 utilizes IL-12Rβ2 and gp130. The precise biological reason for this promiscuity in the use of cytokine and receptor chains is unclear, but this novel feature has implications for the coordinated regulation of this family of cytokines (13). This is especially interesting considering that these cytokines can have opposing effects during an immune response. Broadly, IL-12 and IL-23 are proinflammatory and IL-27 and IL-35 have immunoregulatory functions (13-16).

IL-12 and host resistance to tuberculosis

IL-12 is key mediator of resistance during tuberculosis and is required to promote a protective T_H1 response. One striking example of its importance is a group of genetic disorders characterized by a predisposition to infection with mycobacteria known as Mendelian susceptibility to mycobacterial disease (MSMD). Patients with MSMD frequently exhibit disease in early childhood caused by typically non-pathogenic mycobacteria and often as a result of bacillus Calmette-Guérin (BCG) vaccination (17). MSMD can arise from a variety of genetic mutations, but the most common cause is autosomal recessive complete deficiency of IL-12Rβ1 (18,19). These patients are unresponsive to IL-12 and IL-23 signaling, have blunted IFN-γ production, and often suffer from mycobacterial infections (17,20). Surprisingly, tuberculosis is not particularly common in MSMD patients; however people with *IL12RB1* mutations are the most likely MSDM patients to contract severe forms of tuberculosis (21-24). These finding underscore the protective role of IL-12 family members during mycobacterial infections, including tuberculosis.

In mice, the induction and protective effects of IL-12 are well characterized during tuberculosis. Mtb can directly stimulate IL-12 production by macrophages and DCs in a manner dependent on TLR2 and TLR9 signaling (25-27). This is a key first step in generating a T_H1 response and controlling bacterial growth. Mice lacking MyD88, a major adaptor for TLR signaling, are highly susceptible to Mtb (i.e. higher bacterial burden and decreased survival) and have diminished levels of IL-12p40 and IFN-γ (28,29). Knocking out single TLRs fails to recapitulate the dramatic survival defect seen

in MyD88$^{-/-}$ mice, suggesting some redundancy in signaling. Mice deficient for TLR2, TLR4, or TLR6 are not particularly susceptible to low dose aerosol infection and IL-12p40 production is uncompromised (25,26,30,31). Mice singly deficient for TLR9 have reduced levels of IL-12p40 following aerosol infection; however, dramatic survival defects and increased bacterial burden are only observed when both TLR2 and TLR9 are knocked out (26). In addition to TLR signaling, MyD88 serves as an adaptor for downstream signaling of the IL-1 and IL-18 receptors. IL-18$^{-/-}$ mice show reduced serum levels of IL-12p40 following IV infection with Mtb Erdman (32), and IL-1R$^{-/-}$ macrophages produce less IL-12p40 after H37Rv infection (33). Overall, TLR9 appears to be largely responsible for IL-12p40 production during tuberculosis; however, multiple redundant pathways augment IL-12 levels.

Early studies indicated that exogenous administration of IL-12 reduced bacterial burden and extended survival in mice infected with Mtb (34,35). In these experiments, the protective effect of IL-12 was largely dependent on IFN-γ production, as mice incapable of making IFN-γ were not protected by exogenous IL-12 (36). Studies using knockout mice confirmed the importance of IL-12 in controlling bacterial growth *in vivo*. Following IV infection, mice lacking the β-chain of IL-12, IL-12p40$^{-/-}$, have increased bacterial burden and are incapable of Ag-specific IFN-γ production (37). Later studies demonstrated that infected IL-12p40$^{-/-}$ mice have dramatically reduced numbers of CD4^{+} T cells producing IFN-γ (38). The effects of IL-12 on CD8^{+} T cells have never been directly addressed during tuberculosis.

Of course, IL-12p40 is required to generate both IL-12 and IL-23, and it can form a functional homodimer (IL-12(p40)$_2$) that has both agonistic and antagonistic effects on

IL-12 signaling (39-42). A direct role for IL-23 in contributing to the IFN-γ response was examined in IL-23p19$^{-/-}$ mice. Following aerosol infection, IL-23p19$^{-/-}$ mice mount a robust CD4^{+} T cell response, produce normal levels of IFN-γ, and show no increase in bacterial burden at early time points (43). This experiment demonstrates that IL-23 does not contribute to T_H1 expansion when IL-12 is present.

IL-23 and host resistance to tuberculosis

IL-23 is induced following Mtb infection in a manner largely dependent on TLR2 signaling (44). Though not strictly required for early protective immunity to Mtb, IL-23 can compensate for the loss of IL-12. Mice deficient in the α-chain of IL-12, IL-12p35$^{-/-}$, have increased bacterial burden and decreased survival compared to WT controls; however, they are not as susceptible as IL-12p40$^{-/-}$ mice (38,45). Unlike IL-12p40$^{-/-}$ mice, IL-12p35$^{-/-}$ mice still make IL-23, generate a modest CD4^{+} T cell response, and produce some IFN-γ (38). Thus, IL-23 is capable of supporting a modest T_H1 response in the absence of IL-12 and confers some protection. The susceptibility of the IL-12p40$^{-/-}$ mice is only recreated when mice lack both IL-23p19 and IL-12p35, and are again incapable of producing IL-23 and IL-12 (43). Other models have confirmed the ability of IL-23 to support CD4^{+} T cell function in the absence of IL-12. In IL-12p40$^{-/-}$ mice, co-administration of a plasmid expressing IL-23 can restore the generation of protective IFN-γ-producing T cells in response to DNA vaccination (46).

IL-23 also has independent effects on the immune response during tuberculosis. IL-23 mediates the protective effects of ESAT6-vaccination by inducing a population of IL-17-producing memory cells (47). In fact, eliciting IL-17-producing CD4^{+} T cells (T_H17

cells) in response to Mtb appears to be a major function of IL-23 (43,44). T_H17 cells are important for recruiting cells such as neutrophils to sites of infection. During tuberculosis, they can be protective early on but generate immunopathology if left unchecked (48-51). In this way, IL-23 can have a deleterious effect on the outcome of infection by supporting IL-17-driven immunopathology (48). There is also evidence supporting a protective role for IL-23 during chronic tuberculosis. IL-23p19$^{-/-}$ mice are unable to restrict bacterial growth at later time points during tuberculosis (>150 days), despite maintaining consistent IFN-γ levels (52). The mechanism of this protection is unclear, but the observation illustrates that IL-23 has multiple, often contradictory, effects on susceptibility.

IL-27 and host resistance to tuberculosis

Depending on the context, IL-27 can have both pro- and anti-inflammatory effects, though most recent research has focused on its immunoregulatory functions. In many infections, IL-27 functions to limit immunopathology (16,53). This was first demonstrated in *Toxoplasma gondii* infection, where IL-27 dampens T_H1 responses. IL-27Rα$^{-/-}$ mice succumb early to *T. gondii* infection due to immunopathology as result of a heightened CD4$^+$ T cell response (54). Following Mtb infection, macrophages produce IL-27 and it is also detected in the lungs of infected mice (55,56). During tuberculosis, IL-27Rα$^{-/-}$ mice have reduced bacterial burdens in the lungs associated with the increased recruitment of activated CD4$^+$ T cells (55,56). Hölscher et al. observed increased levels of IL-12p40 and IFN-γ in the lungs of infected IL-27Rα$^{-/-}$ mice and demonstrated that infected IL-27Rα$^{-/-}$ macrophages produce increased amounts IL-

12p40 and TNF (56). Thus, the absence of IL-27 signaling can enhance the immune response to Mtb. Though bacterial growth is better controlled, IL-27Rα$^{-/-}$ mice exhibit extensive immunopathology by 250 days post-infection and die sooner than WT controls (56). This illustrates an essential balance that must be maintained between protective host responses and immunopathology.

IL-12 and IL-27 mediate susceptibility during tuberculosis and form an import axis regulating T_H1 responses. IL-12 is essential for host protection in both mice and humans and promotes a protective CD4$^+$ T cell response. IL-27 serves to dampen this T_H1 response and, in turn, limits immunopathology. It is worth noting that in almost all of the studies described, CD8$^+$ T cells were left unexamined. In IL-27Rα$^{-/-}$ mice, there is increased recruitment of CD8$^+$ T cells to the lungs during tuberculosis, suggesting IL-27 also inhibits CD8$^+$ T cell function (56). However, it is possible that this effect is indirect and results from the lack of CD4$^+$ T cell help. In other infections, direct IL-27 signaling can enhance CD8$^+$ T proliferation and the acquisition of effector functions (57-60). Thus, the full impact of IL-12 and IL-27 on CD8$^+$ T cells during tuberculosis remains to be examined.

Type 1 Interferons

Interferons were discovered in 1957 by Isaacs and Lindenmann and are considered the first cytokine to be identified (61,62). Their name is derived from the initial observation that they “interfere” with viral replication in mammalian cells. Today, there are three interferon (IFN) families: Type 1, Type 2, and Type 3. There are multiple type 1 IFN family members, but the most studied are the IFNα subtypes (14 in humans

and 13 in mice) and the single IFNβ. The lone member of the type 2 family is IFN-γ, and the type 3 family consists of IL-28A, and IL-28B, and IL-29. Nearly every cell in the body can produce type 1 IFN. This is in stark contrast to type 2 IFN (IFN-γ), which is primarily secreted by T cells and NK cells.

The production of type 1 IFN is triggered by a number of pattern recognition receptors (PRRs) found in the cytosol that recognize nucleic acids and other pathogen associated molecular patters (PAMPs) (63-66). Additionally, TLR3, TLR4, TLR7, TLR8, and TLR9 can induce type 1 IFN (63,67). All type 1 IFN family members signal through a ubiquitously expressed heterodimeric receptor consisting of IFNAR1 and IFNAR2 (68). Because most cells can produce and respond to type 1 IFN, this family of cytokines is an especially important in defending against pathogens. However, the effects of type 1 IFN are complex and have both positive and negative effects on host resistance, depending on the pathogen.

Type 1 IFN and susceptibility to tuberculosis

Mtb can trigger type 1 IFN production in infected macrophages through the activation of the cytosolic sensor nucleotide-binding oligomerization domain containing 2 (Nod2) and IFN-β mRNA is detected in mice as soon as 24 hours after IV infection (69,70). During tuberculosis, type 1 IFN is considered detrimental to host resistance, but the precise mechanisms for this negative effect are incompletely understood. Knockout mice lacking a negative regulator of type 1 IFN production, TPL2, have higher bacterial burdens following aerosol infection (71). Mice deficient in the IFNα/β receptor, $IFNAR^{-/-}$, are one of the few knockout strains that better control bacterial growth (70,72,73).

Additionally, IFNAR$^{-/-}$ mice have a survival advantage when infected with hypervirulent clinical isolates (70,72,73). In WT mice, these hypervirulent clinical isolates induce greater amounts of type 1 IFN during infection, and this is associated with a dampened T_H1 response (72-74)

Additional evidence for these negative effects can be found in studies that increase type 1 IFN levels during infection. In short, any factor that augments type 1 IFN production exacerbates disease. Direct administration of either intranasal type 1 IFN or Poly-IC, a potent inducer of type 1 IFN, reduces survival in WT mice (74,75). In a more physiological version of this experiment, co-infection with Mtb and influenza A results in unrestricted Mtb growth, and this phenotype is not observed in co-infected IFNAR$^{-/-}$ mice (76). Decreases T_H1 immunity is a likely mechanism for the negative impact of type 1 IFN; however it is not a universally observed phenomenon in the mouse model (75).

In humans, increased type 1 IFN signaling is associated with active tuberculosis. In a large patient cohort, a 393-gene signature was identified in whole blood that distinguished latent from active disease, and this transcriptional profile diminished as patients underwent antibiotic treatment (77). Pathway analysis revealed that genes downstream of type 1 IFN were overrepresented in this transcriptional signature, thus associating type 1 IFN with an active disease state. Similar associations were made in additional cohorts of patients, confirming this observation (78). Whether increased type 1 IFN is a cause or a result of active disease remains to be determined.

Defining a clear mechanism behind all of these observations is challenging, and it is likely that type 1 IFN exacerbates disease through a number of mechanisms. Early

mouse studies associated a dampened T_H1 response with type 1 IFN overproduction, but it is unclear which cell types are being affected to reduce T cell activation (72,74). Infected macrophages and monocytes respond to increased type 1 IFN by reducing the production of inflammatory cytokines, including IL-12, and simultaneously increase the production of the immunosuppressive cytokine IL-10 (71,79-81). This influence on IL-12 levels has the potential to affect $CD4^+$ T cell expansion and the production of IFN-γ.

In addition to affecting cytokine production, type 1 IFN directly modulates the antimicrobial activity of infected cells in part by diminishing the response to IFN-γ. Type 1 IFN downregulates IFN-γR1 expression on monocytes in addition to inhibiting downstream IFN-γR1 signal transduction (80). In macrophages, IFN-γ-mediated bacterial killing is significantly enhanced when cells lack IFNAR (81). A possible mechanism for this reduction in killing was identified in studies with *Mycobacterium leprae.* In infected macrophages, *M. leprae* inhibits the production of IFN-γ-induced antimicrobial peptides through IFNβ-driven IL-10 production (82). Thus, type 1 IFN directly interferes with the macrophage's ability to kill Mtb. In total, these observations suggest type 1 IFN counters both the production and the cellular response to IFN-γ. Finally, type 1 IFN can affect disease by influencing the recruitment of innate immune cells to the lung. Administration of intranasal Poly-IC during tuberculosis results in the recruitment of a monocyte population ($CD11b^+Gr1^{int}$) that is highly permissive to bacterial growth (75).

Type 1 IFN clearly has the potential to influence adaptive immunity based its ability to suppress IL-12 production. However, no studies have addressed the direct effects of type 1 IFN on $CD4^+$ and $CD8^+$ T cells during tuberculosis. Depending on the

context, type 1 IFN can stimulate or inhibit $CD4^+$ and $CD8^+$ T cell proliferation and effector functions (63). In response to some pathogens, type 1 IFN is a key signal promoting $CD8^+$ T cells expansion, function, and eventual memory formation (discussed in detail below). For this this reason, the effect of type 1 IFN on the $CD8^+$ T cell response to tuberculosis should be examined.

$CD8^+$ T cells require three signals for activation

Classically, the activation of naïve $CD4^+$ and $CD8^+$ T cells is thought to require two signals. The first signal comes from interaction of the T cell receptor (TCR) with its cognate antigen. This antigen must be presented to the naïve T cell in the context of an antigen-presenting molecule on the surface of an APC. In addition to TCR stimulation (signal 1), naïve T cells require signals from a variety of co-stimulatory receptors to efficiently expand and acquire effector functions. CD28 is the prototypical co-stimulatory receptor and is constitutively expressed on both naïve $CD4^+$ and $CD8^+$ T cells. Its ligands, B7.1 and B7.2, are expressed on the surface of APCs and are upregulated upon activation of these cells. While mice lacking either B7.1 or B7.2 alone have no phenotype following Mtb infection, mice lacking both co-stimulatory ligands show delayed T cell priming and have fewer $CD4^+$ and $CD8^+$ T cells producing IFN-γ in their lungs (83,84). These mice are still capable of mounting a modest T cell response, suggesting that other co-stimulatory signals may exist during tuberculosis.

Intriguingly, there is evidence suggesting some microbial products directly co-stimulate T cells. The Mtb lipoprotein LprG can serve as a co-stimulatory signal for human $CD4^+$ T cells in a manner dependent on TLR1 and TLR2 (85), and the TLR2

agonist P3CSK4 is sufficient to co-stimulate transgenic $CD4^+$ T cells specific for Ag85B (86). Rv2468c, a protein primarily found in the Mtb cell wall, can also serve as a co-stimulatory molecule by interacting with the integrin VLA-5 (α5β1) on $CD4^+$ T cells (87). A role for direct costimulation of $CD8^+$ T cells during tuberculosis has not been examined. Co-stimulatory receptors and ligands have a profound influence on T cell biology, and we are only beginning to address the roles these molecules play during tuberculosis.

More recently, the two-signal model of T cell activation has expanded for $CD8^+$ T cells to include a necessary third signal (*Figure 2-1*). The requirement for this third signal was first demonstrated using artificial APCs (aAPCs). These aAPCs are created by incorporating co-stimulatory B7 ligands along with peptide/major histocompatibility complexes (MHC) on the surface of microspheres. This experimental tool has the advantage of minimizing the potential signals delivered to naïve $CD8^+$ T cells. Work with aAPCs *in vitro* revealed that $CD8^+$ T cells require input from inflammatory cytokines in addition to TCR engagement, co-stimulation, and IL-2 during priming (88). The predominant "signal 3" cytokines are IL-12 and type 1 IFN, and they have pivotal roles in influencing $CD8^+$ T cell expansion, differentiation, effector functions, and memory formation (88,89). These effector functions include the production of IFN-γ and the ability to lyse target cells. In the absence of signal 3 cytokines, primed $CD8^+$ T cells can proliferate but fail to develop effector functions and become tolerant to antigen stimulation (90). The first clear *in vivo* examples of a signal 3 came from studies using peptide vaccination in mice. When combined with peptide, IL-12 and type 1 IFN can act in place of an adjuvant to elicit productive $CD8^+$ T cell responses (91-93). This finding

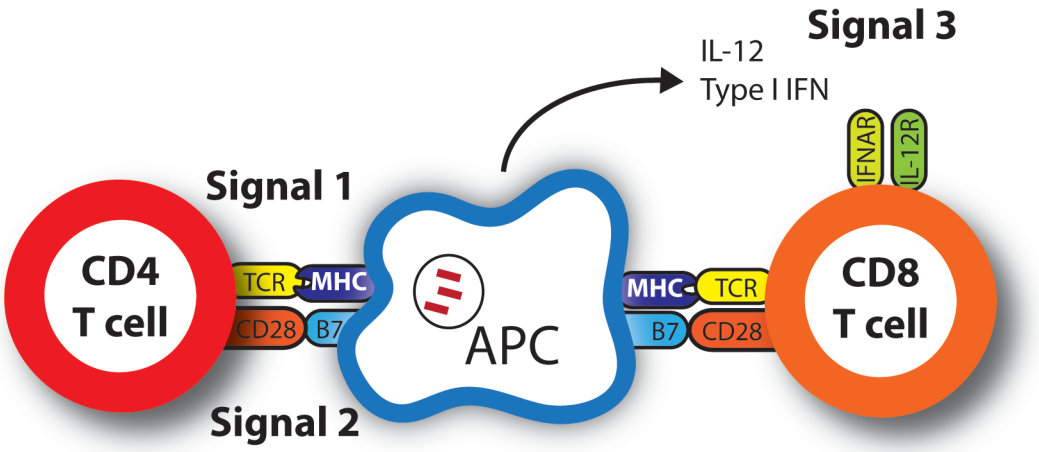

Figure 2-1. IL-12 and Type 1 IFN provide a third signal necessary for efficient CD8$^+$ T cell priming.

In addition to TCR ligation (signal 1), and signals from cell surface co-stimulatory receptors (signal 2), CD8$^+$ T cells require a third signal in the form of an inflammatory cytokine. The predominant signal 3 cytokines are IL-12 and type 1 interferon (type 1 IFN).

suggests that eliciting signal 3 cytokines is an additional component of adjuvant function.

Signal 3 cytokines during infection

The first studies demonstrating a role for signal 3 cytokines during infection were done by transferring naïve TCR transgenic P14 $CD8^+$ T cells into mice and infecting with lymphocytic choriomeningitis virus (LCMV). $IFNAR^{-/-}$ $CD8^+$ cells expand poorly in response to LCMV and can be reduced by as much as 99% relative to WT P14 $CD8^+$ T cells (94,95). In many of these early studies, $IFNAR^{-/-}$ $CD8^+$ T cells initially proliferated following exposure to antigen but ultimately failed to expand as a population (94-96). Recent data have shed light on this phenomenon by demonstrating that signal three cytokines sustain expression of the high-affinity IL-2 receptor, CD25, on primed $CD8^+$ T cells (97). This sustained IL-2 signaling supports a transcriptional program that promotes cell cycle progression genes and facilitates $CD8^+$ T cell expansion.

The requirement for different signal 3 cytokines can differ greatly between pathogens. Direct type 1 IFN signaling is essential for the $CD8^+$ T cell response to LCMV, and IL-12 is unnecessary (96,98). In contrast, IL-12 is essential for $CD8^+$ T cells to acquire effector functions in response to vaccination with an attenuated strain of *T. gondii* (99). To date, *Listeria monocytogenes* (LM) is the only bacterial pathogen examined, and $CD8^+$ T cell expansion is largely dependent on IL-12 production (96,98,100). However, type 1 IFN signaling still supports a small degree of expansion when cells lack IL-12R, indicating that multiple signal 3 cytokines can affect $CD8^+$ T cells in a single infection (98). Though $IL\text{-}12R^{-/-}$ $CD8^+$ T cells expand poorly in response

to *L. monocytogenes*, IFN-γ production and cytolytic activity are only significantly compromised when both IL-12R and IFNAR are absent (98). This suggests that IL-12 and type 1 IFN can compensate for each other to promote effector function.

The relative importance of IL-12 or type 1 IFN during an infection may simply be a matter of the particular cytokines elicited by the pathogen. Keppler and colleagues performed an extensive analysis of IL-12 and type 1 IFN during infections with vaccinia virus (VV), vesicular stomatitis virus (VSV), LCMV, and *L. monocytogenes* (98). Overall, the timing and relative amounts of IL-12 and type 1 IFN elicited during $CD8^+$ T cell expansion correlated with their role as signal 3 cytokines. For example, *L. monocytogenes* induces an initial burst of IL-12 that gives way to increased levels of type 1 IFN. Thus, IL-12 has the biggest impact on $CD8^+$ T cells, while type 1 IFN has a supporting role. During *L.* monocytogenes infection, $CD8^+$ T cell expansion is only fully abrogated when both IL-12R and IFNAR are absent from the $CD8^+$ T cell. It is unclear if a single $CD8^+$ T cell responds to both IL-12 and Type 1 IFN or if there are distinct populations of cells responding to a single cytokine during infection. It should be noted that some pathogens elicit strong $CD8^+$ T cell response without a known signal 3. The $CD8^+$ T cell response to VV is unaltered even in the absence of both IL-12R and IFNAR (98,101). This is surprising given that VV elicits IL-12 production and suggests that additional sources of signal 3 exist.

Signal 3 cytokines regulate the differentiation of effector $CD8^+$ T cells.

Beyond promoting expansion and effector function, signal 3 cytokines are important regulators of $CD8^+$ T cell differentiation during infection. Following priming,

$CD8^+$ T cells can adopt several phenotypes that ultimately determine which cells will progress to form a memory population. This was first demonstrated in *L. monocytogenes* infection, where long-lived memory cells are preferentially derived from a small subset of effector cells expressing the IL-7 receptor subunit α (CD127) (102-104). These $CD127^{hi}$ cells are now termed memory precursor effector cells (MPECs). The majority of $CD127^{lo}$ effectors cells are shorter lived and much of this population dies following the clearance of infection. These short-lived effector cells (SLECs) express high amounts of killer cell lectin-like receptor G1 (KLRG1). Cells that express neither KLRG1 or CD127 are early effector cells (EECs) and have yet to adopt a SLEC or MPEC phenotype (105-108).

KLRG1 and CD127 can be useful markers to identify SLECs ($KLRG1^{hi}$ $CD127^{lo}$) and MPECs ($KLRG1^{lo}$ $CD127^{hi}$); however, they are not absolute determinants of a cell's memory potential. For example, some $KLRG1^{hi}$ cells survive to become memory cells and can mount highly protective secondary responses (107,109). Additionally, $KLRG1^{hi}$ $CD127^{hi}$ (termed double positive effector cells – DPECs) exist during most infections and have a survival half-life between SLECs and MPECs (107). An individual $CD8^+$ T cell can show remarkable heterogeneity in its ability to form each of these populations (EEC, SLEC, DPEC, and MPEC), and we are only beginning to understand the factors involved in these cell fate decisions. It appears that the inflammatory milieu elicited by different pathogens plays some role in determining the relative proportion of these effector populations (108,110).

In general, signal 3 cytokines drive the terminal differentiation of $CD8^+$ T cells and promote KLRG1 expression. This is especially true of IL-12 which drives SLEC

differentiation by promoting the expression of the transcription factor T-bet (103). This increased formation of SLECs occurs at the expense of MPEC generation, suggesting that heightened inflammation undermines memory formation by promoting the terminal differentiation of cells. Indeed, some studies show that limiting inflammation during infection can enhance memory formation (111). Similarly, blocking signal 3 during infection can promote the formation of MPECs. IL-12R$^{-/-}$ CD8^{+} T cells overwhelming adopt an MPEC phenotype following *L. monocytogenes* infection, and similar observations have been made with other pathogens (96,98). However, it is unclear if these IL-12R$^{-/-}$ MPECs are functional memory cells. Constitutive expression of CD127 is insufficient to support memory cell formation, suggesting that CD127 alone cannot define true memory potential (112).

There is substantial evidence that signal 3 cytokines are required for the formation of memory cells. CD8^{+} T cells stimulated *in vitro* with aAPCs and then transferred into mice only survive to form a memory population when IL-12 is included with the initial stimulation (100). Studies of memory recall responses have also demonstrated a clear requirement for signal 3 cytokines (113). Signal 3 cytokines control a balance between SLEC and MPEC phenotypes by favoring SLEC differentiation; however, functional memory cannot be formed in the absence of signal three.

IL-27 as an alternative signal 3 cytokine

Though it has never been directly associated with signal 3 activity, IL-27 can affect CD8^{+} T cell function in ways similar to IL-12 and type 1 IFN. Obar and colleagues

demonstrated that IL-27 is important for the accumulation of SLECs during VSV infection, and cells are more likely to adopt an MPEC phenotype in its absence (108). Surprisingly, $CD8^+$ T cell expansion was not affected by the loss of IL-27 in this system. This ability to promote SLEC differentiation likely results from IL-27 inducing T-bet expression (57). IL-27 is also associated with promoting $CD8^+$ T cell function. IL-27 is required to promote IFN-γ expression during both *T. gondii* and influenza virus infection (58). *In vitro*, IL-27 augments the expression of effector molecules such as granzyme B and perforin in naïve $CD8^+$ T cells stimulated with anti-CD3 and anti-CD28 (57,59). Recently, the role of IL-27 was examined in a peptide vaccination model using Poly-IC and anti-CD40 as adjuvants. In this model, $CD8^+$ T cells required IL-27 for both primary expansion and recall responses (114). Furthermore, the transfer of vaccine-elicited IL-$27R^{-/-}$ $CD8^+$ T cells was unable to protect against *L. monocytogenes* infection. These observations suggest IL-27 has an important role in augmenting $CD8^+$ T cells response during infection, and other groups have speculated that it may be an alternative signal 3 cytokine (98).

Summary

IL-12, type 1 IFN, and IL-27 are all elicited during tuberculosis and have profound effects on disease outcome. IL-12 is required for host resistance in mice and humans, and has an essential role in promoting $CD4^+$ T cell responses. In contrast, IL-27 acts as an immunoregulatory cytokine and can dampen $CD4^+$ T cell responses. During tuberculosis, IL-27 limits the control of bacterial growth but is necessary to prevent immunopathology during chronic disease. Type 1 IFN has a variety of effects during

infection, and its overproduction is detrimental to host resistance. The increased resistance of IFNAR$^{-/-}$ mice to Mtb infection underscores this fact. A similar association exists in humans, where type 1 IFN signaling is linked to active disease.

In other infections, all three of these cytokines directly regulate CD8^{+} T cells and can act as essential signals promoting CD8^{+} T cell expansion and effector function. However, the effects of these cytokine on CD8^{+} T cells during tuberculosis are uncharacterized. In fact, little is known about the inflammatory signals governing CD8^{+} T function during tuberculosis. Here, we seek to identify the cytokines that augment or potentially inhibit CD8^{+} T cells, and begin by addressing the roles of IL-12, type 1 IFN, and IL-27. Given the important role of IL-12 in promoting CD4^{+} T cell responses during tuberculosis, we hypothesized IL-12 will also be a dominant signal for CD8^{+} T cell expansion and function. The roles of type 1 IFN and IL-27 are more challenging to predict, and they potentially have a variety of effects.

Using 1:1 mixed bone marrow chimeras, we demonstrate that IL-12 is essential to promote CD8^{+} expansion and the acquisition of effector function. However, type 1 IFN and IL-27 are not dispensable and also augment the expansion of effector cells. These findings support a model in which each cytokine influences CD8^{+} T cell expansion in a non-redundant way. Using bone marrow chimeras, we interrogate the cytolytic ability of IL-12R$^{-/-}$, IFNAR$^{-/-}$, and IL-27R$^{-/-}$ CD8^{+} T cell *in vivo*. Overall specific killing is reduced in the absence of IL-12; however, even IL-12R$^{-/-}$ CD8^{+} T cells remain highly cytolytic. This surprising finding indicates that cytolysis is a robust effector function during tuberculosis and is likely promoted and executed through redundant mechanisms.

Using the adoptive transfer of retrogenic Ag-specific $CD8^+$ T cells, we directly examine priming following low-dose aerosol infection. These studies reveal that IL-12 is necessary to prime $CD8^+$ T cells in the lymph node and to continue their expansion in the lungs. For this reason, IL-12 is the dominant signal 3 cytokine during tuberculosis. In total, IL-12 promotes $CD8^+$ T cell priming, expansion, SLEC differentiation, and IFN-γ production, while type 1 IFN and IL-27 support expansion in the lungs. To date, this is the most comprehensive study of $CD8^+$ T cell regulation during tuberculosis. We believe such understanding has potential implications for rational vaccine design and the development of immunotherapies.

Results

$CD8^+$ T cells express the receptors for IL-12, type 1 IFN, and IL-27 throughout Mtb infection.

To determine the expression of IL-12R, IFNAR, and IL-27R on $CD8^+$ T cells during Mtb infection, we used a method based on the protocol set forth by the Immunological Genome Project (115). Briefly, magnetic bead purified T cells were pooled from the lungs of naïve or infected mice and sorted by FACS to obtain a population of 99% pure $CD3^+$ $CD8^+$ cells. Gene expression profiling was done using the Affymetrix Mouse Gene 1.0ST array and relative expression values were normalized across all the samples in the experiment. Here, we are interested in the relative expression of the subunits for the IL-12 receptor (*Il12rb1* and *Il12rb2*) the interferon-α/β receptor (*Ifnar1* and *Ifnar2*) and the IL-27 receptor (*Il27ra* and *Il6st*). *IL12rb1* transcripts were marginally detectable in cells from uninfected mice (time 0) and *IL12rb2* was undetectable (*Figure 2-2A*). Following infection, transcription of both IL-12 receptor subunits increased and remained detectable throughout the experiment (*Figure 2-2A*). Expression of the interferon-α/β and IL-27 receptor subunits remained relatively high in $CD8^+$ T cells in both uninfected and infected mice (*Figure 2-2A*). IL-12p40 and IL-27 levels were also quantified in lung homogenates from mice infected for 0, 1, 2, and 4 weeks (*Figure 2-2B*). IL-12p40 was undetectable in the lungs of uninfected mice and steadily increased through the first four weeks of infection. IL-27 was detectable in uninfected mice and began to rise 2 weeks after infection. IFNβ is detectable in bronchoalveolar lavage fluid from the lungs of infected mice and was previously

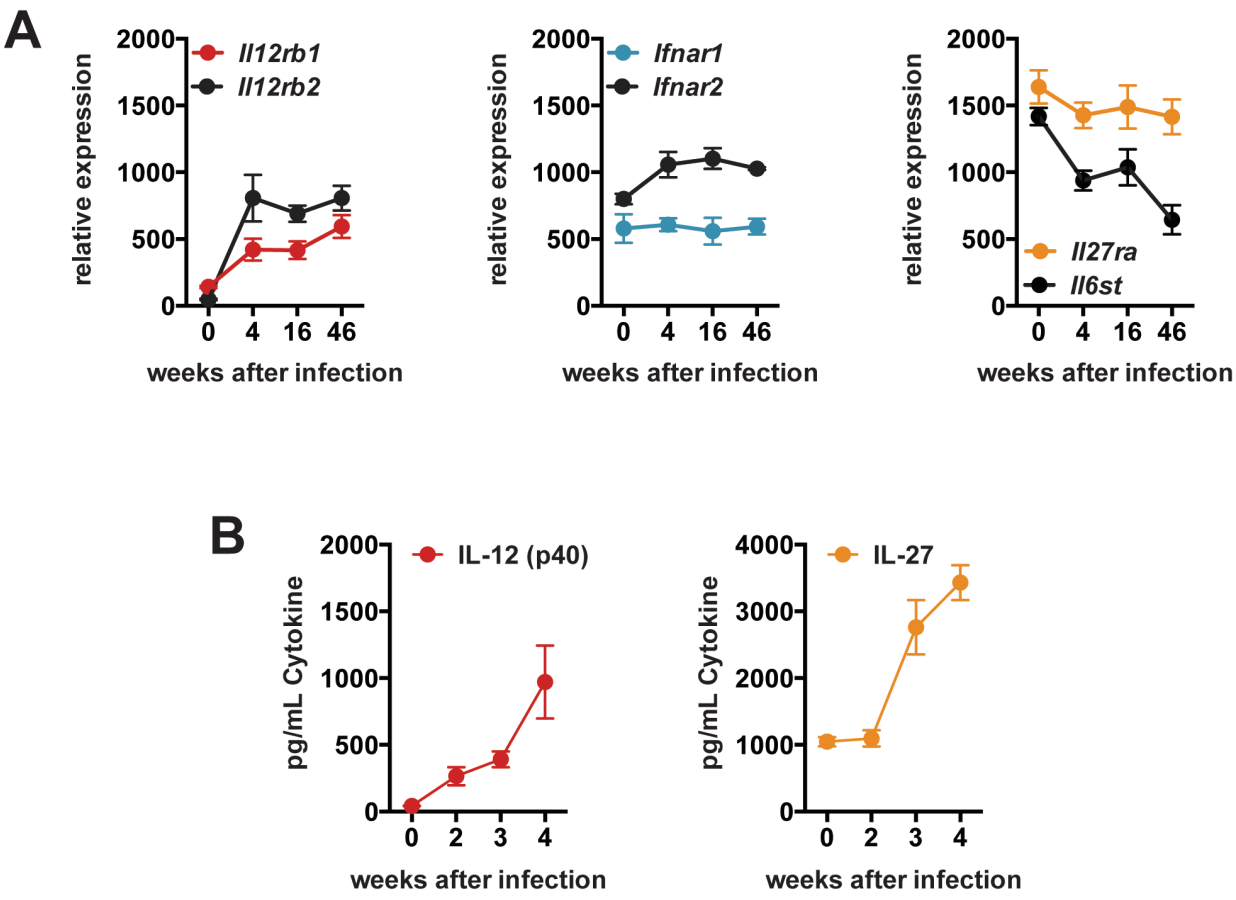

Figure 2-2. CD8$^+$ T cells express the receptors for IL-12, type 1 IFN, and IL-27 throughout Mtb infection.

(A) Relative expression of the indicated cytokine receptor subunits on sorted CD8$^+$ T cells purified from the lungs of uninfected mice (time 0) as well as 4, 16, and 46 weeks after low dose aerosol infection. At each time point, lung homogenates were pooled from three mice prior to magnetic bead purification and cell sorting. Replicates reflect three independent infections, each with three pooled mice. (B) Cytokine array data from lung homogenates of infected mice. N=5 mice per time point. For relative expression, values >120 have a 95% probability or greater of true expression and values <47 have 95% probability or greater of being silent.

reported (116). Together, these findings suggest that $CD8^+$ T cells are capable of responding to all three cytokines throughout infection.

IL-12, type 1 IFN, and IL-27 augment the magnitude of the $CD8^+$ T cell response during tuberculosis.

Previous studies examined the roles of IL-12, type 1 IFN, and IL-27 during tuberculosis by directly infecting knockout mice lacking either the functional cytokines or the relevant receptors. The inflammatory response and degree of susceptibility to Mtb varies substantially between these various knockout strains thus confounding a direct comparison of these experiments. To determine the effects of IL-12, type 1 IFN, and IL-27 on $CD8^+$ T cell responses during tuberculosis, we utilized 1:1 mixed bone marrow chimeras in an aerosol infection model, which allowed for the direct comparison of wild-type (WT) and receptor knock-out (KO) $CD8^+$ T cells within the same host mouse. This experimental system has the key advantage of exposing both WT and KO $CD8^+$ T cells to the same inflammatory environment and bacterial burden throughout the infection.

To generate chimeric mice, congenically marked recipients ($CD90.1^+$) were lethally irradiated and reconstituted with equal ratios of WT ($CD45.1^+$) bone marrow and bone marrow from one of the receptor knockout strains ($CD45.2^+$) (*Figure 2-3A*). The donor knockout mice lacked one of following cytokine receptors: IL-12 receptor beta 2 ($IL\text{-}12R^{-/-}$), interferon-α/β receptor 1 ($IFNAR^{-/-}$), or IL-27 receptor alpha ($IL\text{-}27R^{-/-}$). After reconstitution, the resulting chimeras had equivalent ratios of WT and KO $CD4^+$ and $CD8^+$ T cells in the peripheral blood, lungs, and spleen (*Figure 2-3B and 2-3C, and data not shown*). This suggests that the absence of the individual cytokine receptors did not

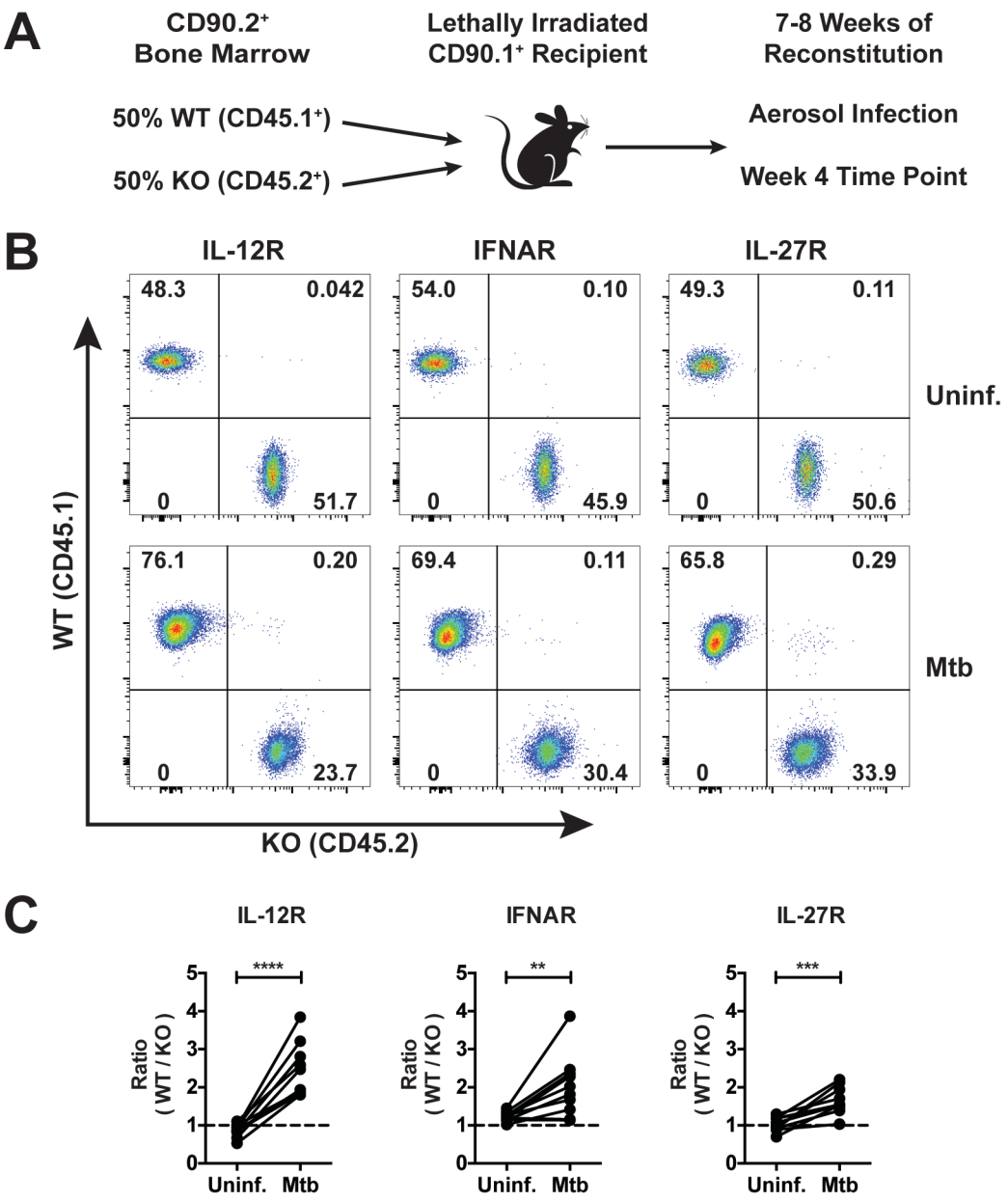

Figure 2-3. IL-12, type 1 IFN, and IL-27 augment CD8$^+$ T cell expansion following Mtb infection.

(A) Overview of 1:1 mixed bone marrow chimera generation. The WT:KO combinations are: WT:IL-12R$^{-/-}$; WT:IFNAR$^{-/-}$; and WT:IL-27R$^{-/-}$. (B) Representative flow cytometry plots of CD3$^+$ CD8$^+$ T cells from the blood of the same chimeric mouse before and four weeks after infection with Mtb. (C) The ratio of WT to KO CD8$^+$ T cells in the indicated 1:1 chimeric mice before and after infection (n = 9-10 mice per group). **P < 0.01, ***P < 0.001, ****P < 0.0001 (paired Student's t-test). Data are representative of three independent experiments.

significantly alter T cell development and homeostasis in uninfected mice. Once baseline reconstitution was assessed, the chimeras were infected via the aerosol route and examined four weeks later at the peak of the adaptive immune response. In control experiments, we generated 1:1 mixed bone marrow chimeras with a mixture of WT CD45.1$^+$ and WT CD45.2$^+$ bone marrow. These control mice maintained an equal ratio of CD45.1$^+$ and CD45.2$^+$ CD8$^+$ T cells in the blood and lungs four weeks following infection with Mtb (*Figure 2-4*), confirming that both groups of donor-derived cells respond to infection.

By determining the ratio of WT and KO CD8$^+$ T cells in the blood, we tracked the proportions of WT and KO CD8$^+$ T cells in the same mice before and after infection with Mtb. Four weeks after infection, IL-12R$^{-/-}$, IFNAR$^{-/-}$, and IL-27R$^{-/-}$ CD8$^+$ T cells were underrepresented in the blood relative to WT cells (*Figure 2-3B and 2-3C*). This was also true in the lungs of infected mice, where the percentage and number of IL-12R$^{-/-}$, IFNAR$^{-/-}$, and IL-27R$^{-/-}$ CD8$^+$ T cells were reduced relative to their WT counterparts in the same mouse (*Figure 2-5A and 2-5B*). Tetramer staining of CD8$^+$ T cells specific for the immunodominant antigen TB10.4$_{4-11}$ (ESXH) revealed the same requirement for all three cytokines in maintaining antigen specific cells in the lungs, and KO CD8$^+$ T cells comprised a significantly smaller percentage of the antigen-specific population (*Figure 2-5C*). Overall, these data indicate that all three cytokines (IL-12, type 1 IFN, and IL-27) are necessary for the accumulation of CD8$^+$ T cells in the lungs during tuberculosis. Of these cytokines, IL-12 appears to have the greatest impact, as IL-12R$^{-/-}$ CD8$^+$ T cells demonstrated the most dramatic reduction in cell numbers. However, type 1 IFN and IL-27 clearly have roles augmenting the magnitude of the CD8$^+$ T cell response.

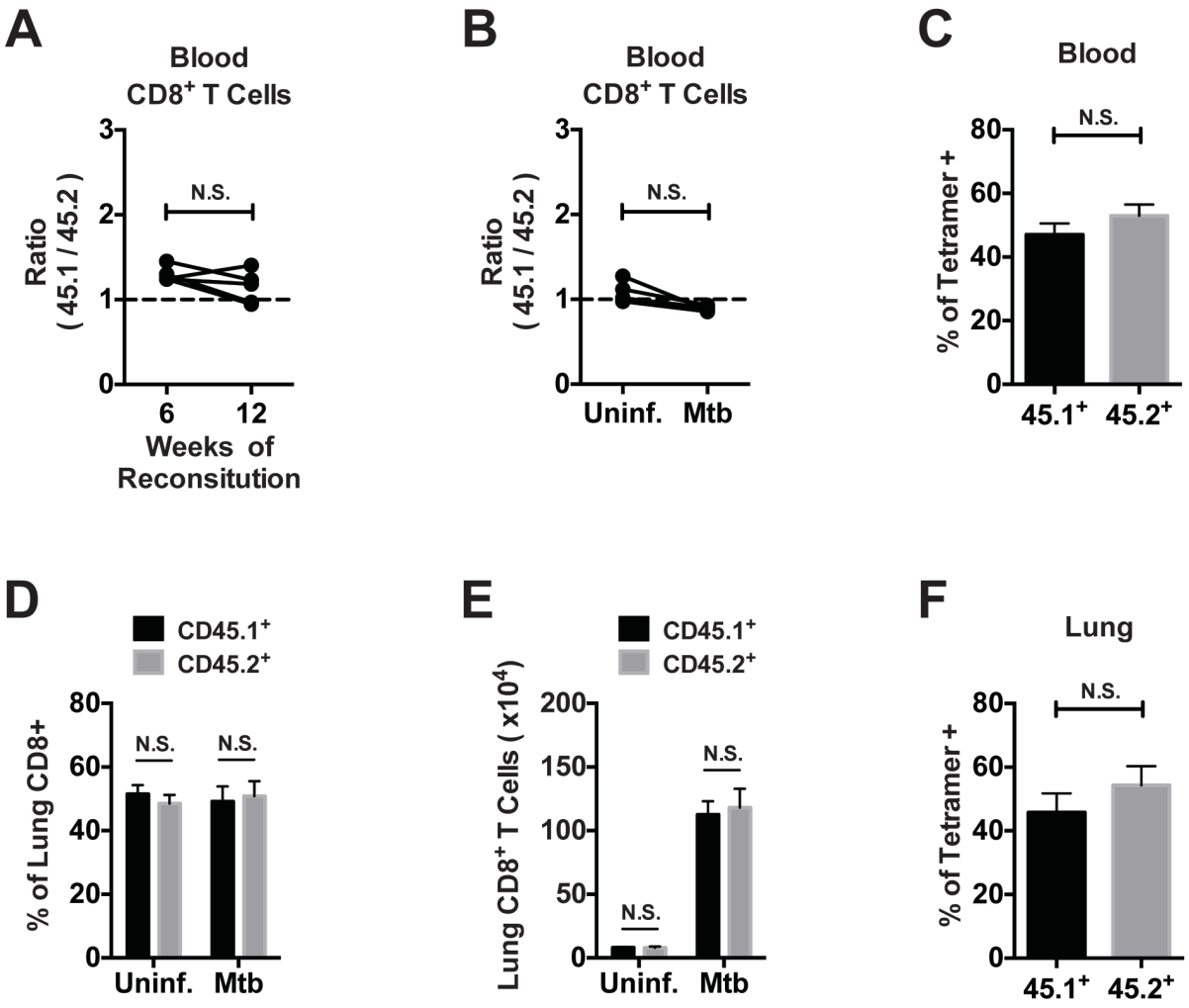

Figure 2-4. Both groups of donor bone marrow can contribute to the immune response following Mtb infection in 1:1 mixed bone marrow chimeras.

Here, a control group of bone marrow chimeras was generated using a 1:1 mixture of WT CD45.1$^+$ and WT CD45.2$^+$ bone marrow. (A) The ratio of CD45.1$^+$ to CD45.2$^+$ CD8$^+$ T cells after 6 and 12 weeks of reconstitution in uninfected mice. (B) The ratio of CD45.1$^+$ to CD45.2$^+$ CD8$^+$ T cells before and four weeks after Mtb infection. (C) The percentage of antigen-specific CD8$^+$ T cells positive for CD45.1 or CD45.2 in the blood. (D) The percentage CD8$^+$ T cells positive for CD45.1 or CD45.2 in the lungs before and after infection. (E) Total number CD45.1$^+$ and CD45.2$^+$ CD8$^+$ T cells in the lungs of uninfected mice and mice 4 weeks post-infection. (F) The percentage of antigen-specific CD8$^+$ T cells positive for CD45.1 or CD45.2 in the lungs of infected chimeras. The tetramer used to determine antigen-specific CD8$^+$ T cells contains the immunodominant epitope TB10.4$_{4-11}$. Each bar represents the mean ± SEM (n = 4-5 mice per group). N.S. = not significant (paired Student's t-test). Data are representative of two independent experiments.

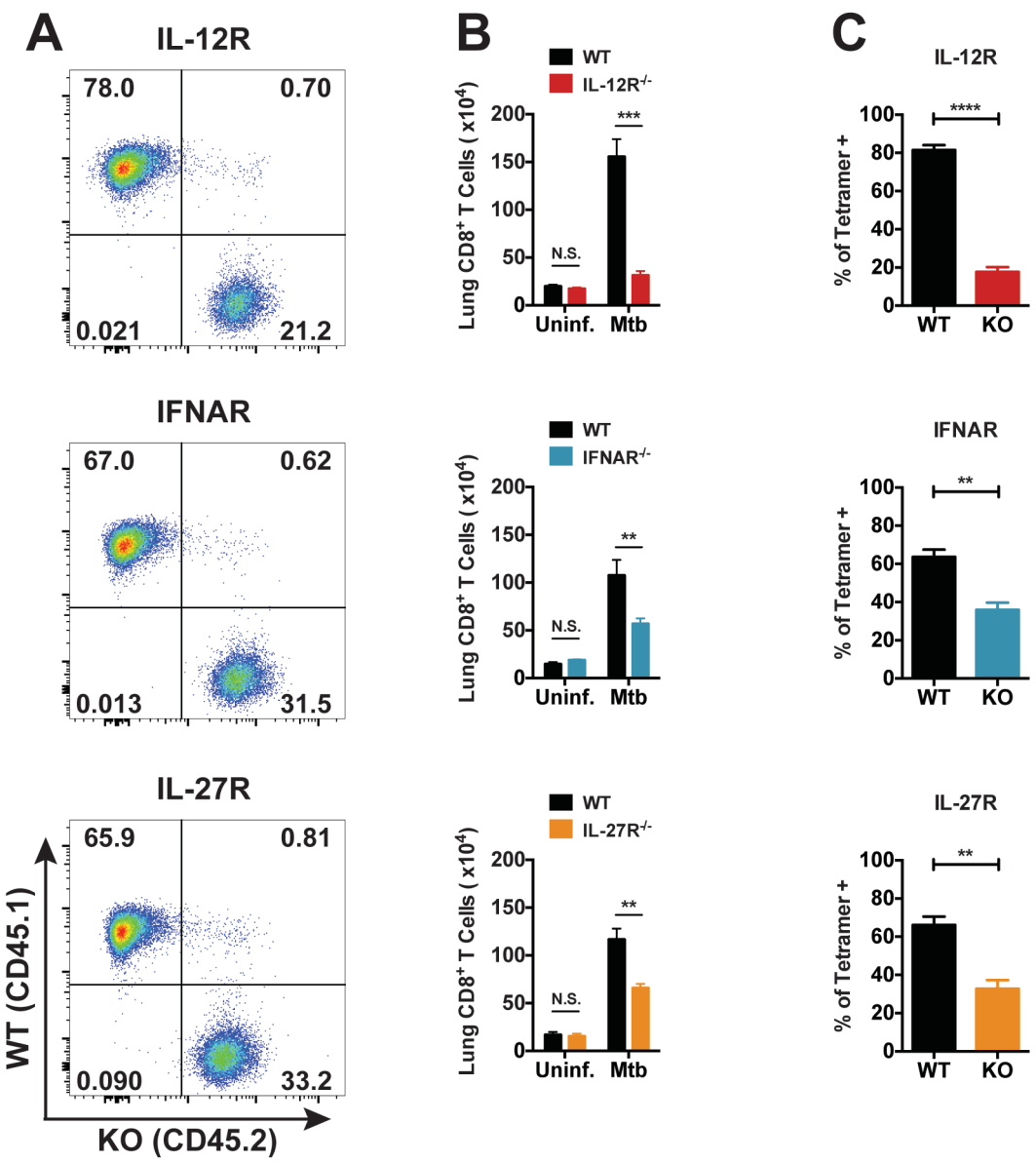

Figure 2-5. IL-12, type 1 IFN, and IL-27 are required for the efficient accumulation of CD8$^+$ T cells in the lungs following Mtb infection.

(A) Representative flow cytometry plots of CD3$^+$ CD8$^+$ T cells from the lungs of the indicated chimeric mice 4 weeks after infection. (B) Total number of WT (CD45.1$^+$) or KO (CD45.2$^+$) CD8$^+$ T cells in the lungs of uninfected chimeric mice and mice 4 weeks post-infection. (C) The percentage of TB10.4-specific CD8$^+$ T cells that are WT or KO in the lungs of infected chimeras. Each bar represents the mean ± SEM (n = 4-5 mice per group for the uninfected groups and n = 8-10 mice per group for the infected groups) **P < 0.01, ***P < 0.001, ****P < 0.0001 (paired Student's t-test). Data are representative of three independent experiments for the infected groups and two independent experiments for the uninfected.

Both IL-12 and IL-27 influence the differentiation of effector $CD8^+$ T cells during tuberculosis.

Following priming, $CD8^+$ T cells can differentiate into several distinct effector subpopulations that are distinguishable by their expression of the cell surface markers killer cell lectin-like receptor subfamily G, member 1 (KLRG1) and interleukin-7 receptor (CD127) (103,104). Recently primed effector cells lack expression of both KLRG1 and CD127 and are known as early effector cells (EECs) (108). These cells then give rise to the two main effector subpopulations: short-lived effector cells (SLEC – $KLRG1^{Hi}$ $CD127^{Lo}$) and memory precursor effector cells (MPEC – $KLRG1^{Lo}$ $CD127^{Hi}$). MPECs are the population that will eventually give rise to long-lived memory cells. A fourth subpopulation exits that expresses both KLRG1 and CD127 (DPEC – double positive effector cell); however, the functional relevance of these cells remains unclear.

The inflammatory environment elicited by the pathogen influences these cell fate decisions, and IL-12, type 1 IFN and IL-27 all have differing roles in $CD8^+$ T cell differentiation during different infections (98,110). We examined the role of these cytokines in $CD8^+$ T cell differentiation during tuberculosis using the 1:1 mixed bone marrow chimera model. Four weeks post infection, antigen-specific cells were determined by staining with $TB10.4_{4\text{-}11}$ tetramers, and the expression of KLRG1 and CD127 was then evaluated in the WT and receptor knockout populations. Following Mtb infection, WT $CD8^+$ T cells primarily adopt a SLEC and EEC phenotype with few cells expressing CD127 (*Figure 2-6A and 2-6B*). Loss of IL-12 signaling severely skewed the differentiation of effector cells, resulting in a substantial decrease in terminally differentiated SLECs and a subsequent increase in both EECs and MPECs

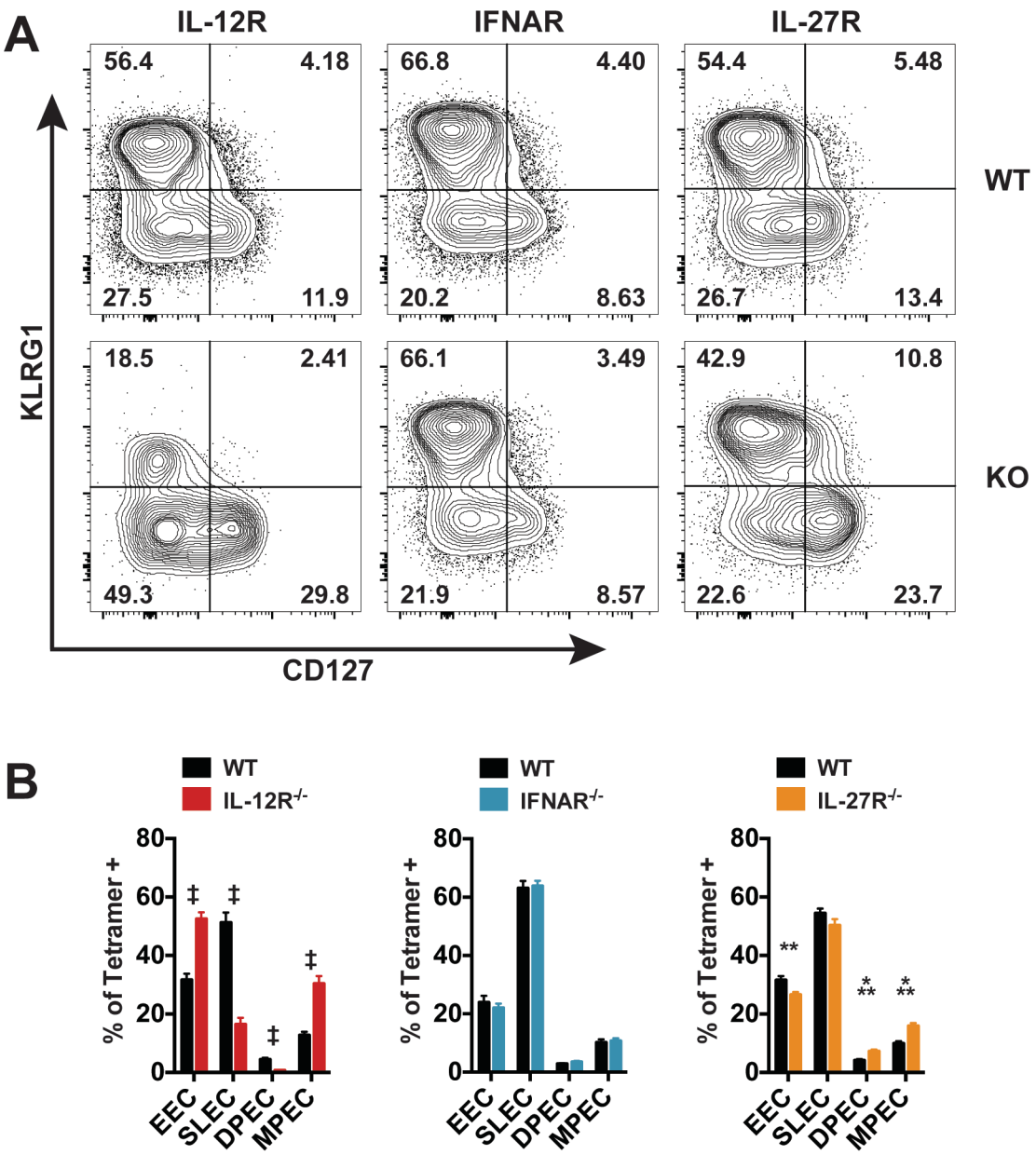

Figure 2-6. IL-12 and IL-27 influence the differentiation of effector CD8$^+$ T cells during tuberculosis.

(A) Representative flow cytometry plots of lung CD8$^+$ TB10.4$^+$ T cells of the indicated genotype 4 weeks after infection in 1:1 mixed bone marrow chimeras. (B) Phenotypic analysis of CD8$^+$ TB10.4$^+$ cells based on KLRG1 and CD127 staining (EEC – Early Effector Cells; SLEC – Short-Lived Effector Cells; DPEC – Double Positive Effector Cell; MPEC – Memory Precursor Effector Cell) Each bar represents the mean ± SEM (n = 9-10 mice per group) **P < 0.01, ***P < 0.001, ‡P < 0.0001 (paired Student's t-test). Data are representative of three independent experiments.

(*Figure 2-6A and 2-6B*). Loss of type 1 IFN signaling had no impact on the relative proportion of effector subpopulations (*Figure 2-6A and 2-6B*). The absence of IL-27 signaling lead to increased expression of CD127 accompanied by a mild decrease in EECs (*Figure 2-6A and 2-6B*). Frequently, an increase in MPECs is accompanied by decreased SLEC generation (98,108); however, SLECs accumulated normally without IL-27R. Of the cytokines examined, IL-12 appears to be a main determinant of $CD8^+$ T cell differentiation during tuberculosis, as its absence resulted in the most substantial skewing of effector subpopulations.

IL-12, type 1 IFN, and IL-27 have distinct and overlapping effects on $CD8^+$ T cell function during tuberculosis.

IL-12, type 1 IFN, and IL-27 can all impact the acquisition of effector functions by $CD8^+$ T cells, but their effects on $CD8^+$ T cell function during tuberculosis has not been examined. Interferon gamma (IFN-γ) is a particularly important cytokine during tuberculosis, and $CD8^+$ T cells are a source of this protective cytokine. We stimulated lung cells from 1:1 mixed bone marrow chimeras *ex vivo* with the immunodominant $TB10.4_{4\text{-}11}$ peptide and performed intracellular cytokine staining to assess the percentage of cells producing IFN-γ. As controls, lung cells were also left unstimulated or stimulated with anti-CD3 and anti-CD28. Significant levels of cytokine production were not observed in the unstimulated samples (data not shown). Of the three cytokines, IL-12 was the only one essential for IFN-γ production by lung $CD8^+$ T cells four weeks after infection (*Figure 2-7A and 2-7B*). Compared to WT IFN-γ^+ $CD8^+$ T cells, the IL-$12R^{-/-}$ IFN-γ^+ $CD8^+$ T cells also had a significantly reduced MFI, indicating

Figure 2-7. IL-12 promotes $CD8^+$ T cell derived IFN-γ production.

(A) Representative flow cytometry plots of lung $CD8^+$ T cells of the indicated genotype 4 weeks after infection. Lung homogenates were stimulated ex vivo with TB10.4 peptide prior to IFN-γ ICS. (B) The percentage of WT and KO $CD8^+$ T cells positive for IFN-γ as determined by ICS. Lung cells were stimulated ex vivo with either TB10.4 peptide or αCD3 and αCD28. (C) IFN-γ MFI of the IFN-$γ^+$ populations from lung homogenates stimulated ex vivo with TB10.4 peptide (same samples as panel B). Each bar represents the mean ± SEM (n = 9-10 mice per group) N.S. = Not Significant, **P < 0.01, ***P < 0.001, ****P < 0.0001 (paired Student's t-test). Data are representative of three independent experiments.

Figure panels on following page.

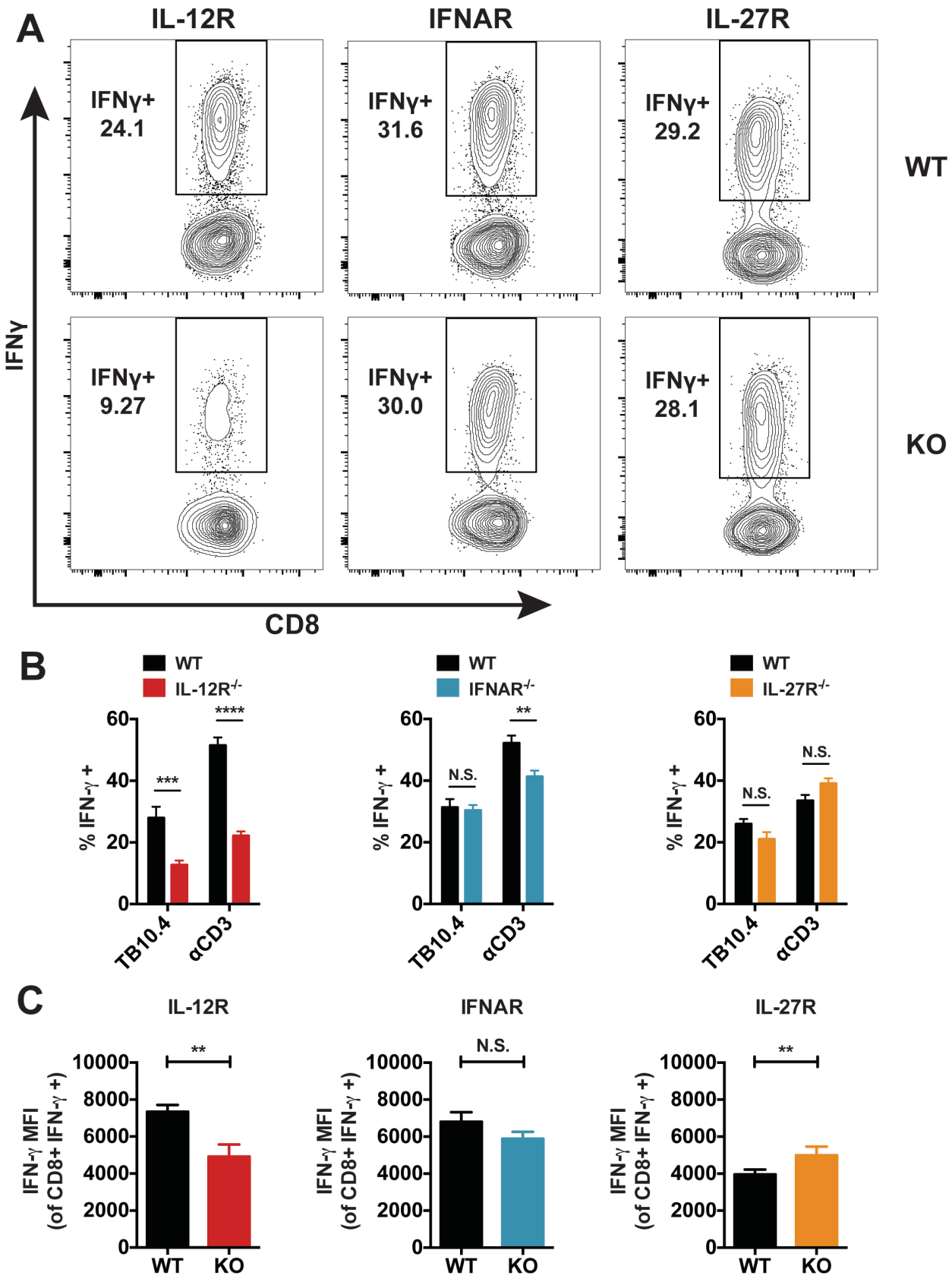

Figure 2-7 (Continued). IL-12 promotes CD8$^+$ T cell derived IFN-γ production.

that IFN-γ^{+} cells were making less cytokine in the absence of IL-12 signaling (*Figure 2-7C*). Surprisingly, IL-27R$^{-/-}$ IFN-γ^{+} CD8^{+} T cells had a significantly higher MFI, suggesting that IL-27R$^{-/-}$ CD8^{+} T cells may produce more IFN-γ on a per cell basis. (*Figure 2-7C*). This may be analogous to the increase in IFN-γ production observed in CD4^{+} T cells from IL-27R$^{-/-}$ mice following Mtb infection (56).

In addition to cytokine production, IL-12, type 1 IFN, and IL-27 can alter the cytolytic capacity of CD8^{+} T cells. To address the role of these cytokines in inducing cytolytic function, we analyzed granzyme B levels in CD8^{+} T cells from the lungs of 1:1 mixed bone marrow chimeras. Surprisingly, the loss of receptor signaling for all three cytokines resulted in decreased levels of granzyme B by both percentage and overall MFI (*Figure 2-8A and 2-8B*). These findings lead us to hypothesize that all three cytokines are required for optimal cytolytic function.

Following Mtb infection, elicited CD8^{+} T cells remain cytolytic in the absence of IL-12, type 1 IFN, or IL-27 signaling.

With the goal of examining cytolytic function, we adopted a new strategy to generate bone marrow chimeras in which all the CD8^{+} T cells lacked the cytokine receptors of interest (*Figure 2-9A*). Briefly, TCRα$^{-/-}$ mice were lethally irradiated and reconstituted with a mixture of donor cells consisting of 80% CD8α$^{-/-}$ bone marrow and 20% of either WT or receptor knockout bone marrow (IL-12R$^{-/-}$, IFNAR$^{-/-}$, or IL-27R$^{-/-}$). In the resulting “4:1” chimeric mice, CD8^{+} T cells can only be derived from either the donor WT or receptor knockout bone marrow, depending on the experimental group. CD8^{+} T cells developed normally in these mice and responded robustly to aerosol infection with

Figure 2-8. Multiple cytokines augment granzyme B production.

(A) Representative flow cytometry plots of lung $CD8^+$ T cells of the indicated genotype 4 weeks after infection. Lung homogenates were stimulated ex vivo with TB10.4 peptide prior to intracellular staining for granzyme B. (B) The percentage of WT and KO $CD8^+$ T cells positive for intracellular granzyme B staining from lung cells stimulated ex vivo with TB10.4 peptide. (C) MFI of total granzyme B staining in WT and KO $CD8^+$ T cells from lung cells stimulated ex vivo with TB10.4 peptide (same samples as panel B). Each bar represents the mean ± SEM (n = 9-10 mice per group) *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (paired Student's t-test). Data are representative of three independent experiments.

Figure panels on following page.

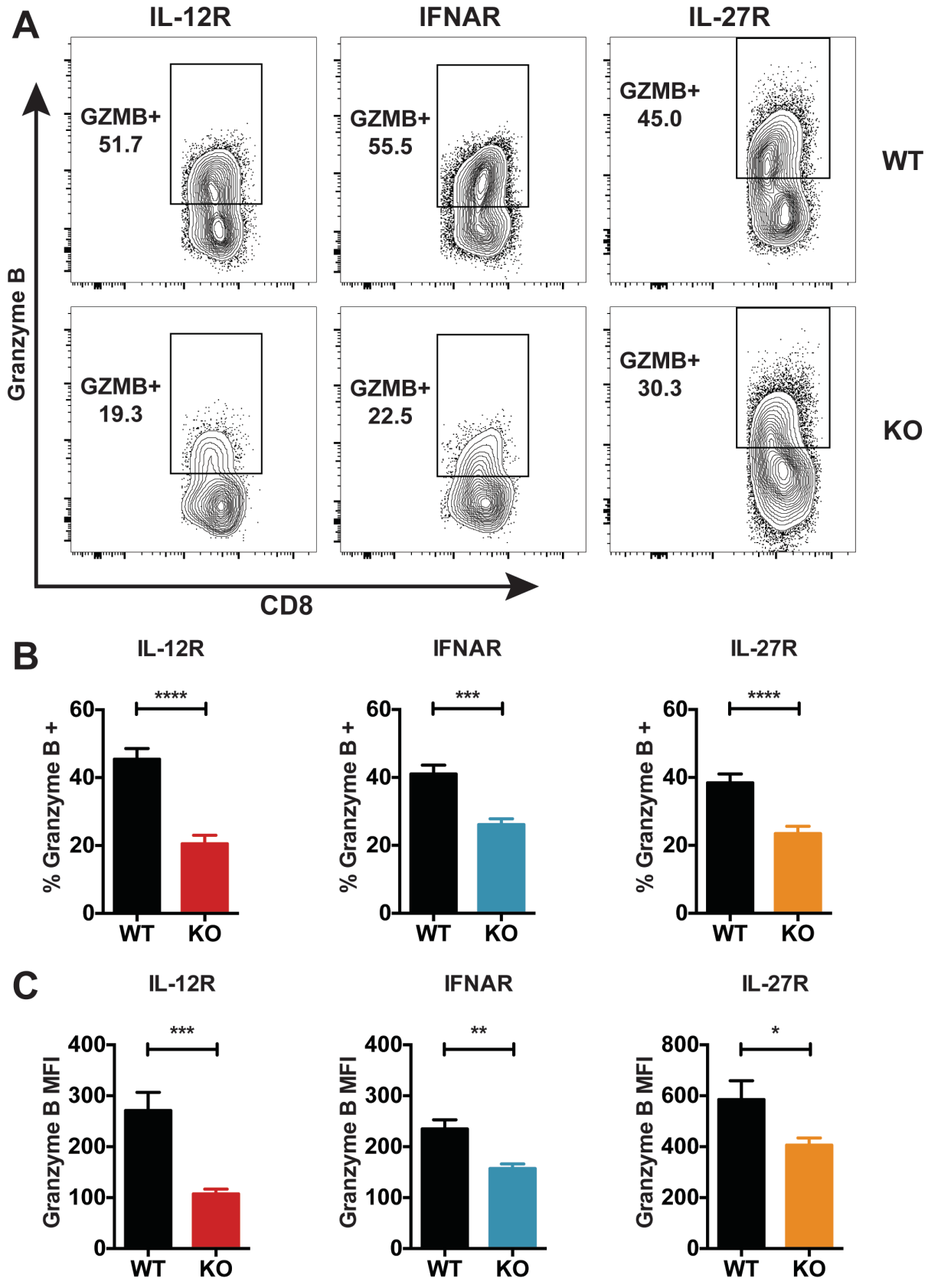

Figure 2-8 (Continued). Multiple cytokines augment granzyme B production.

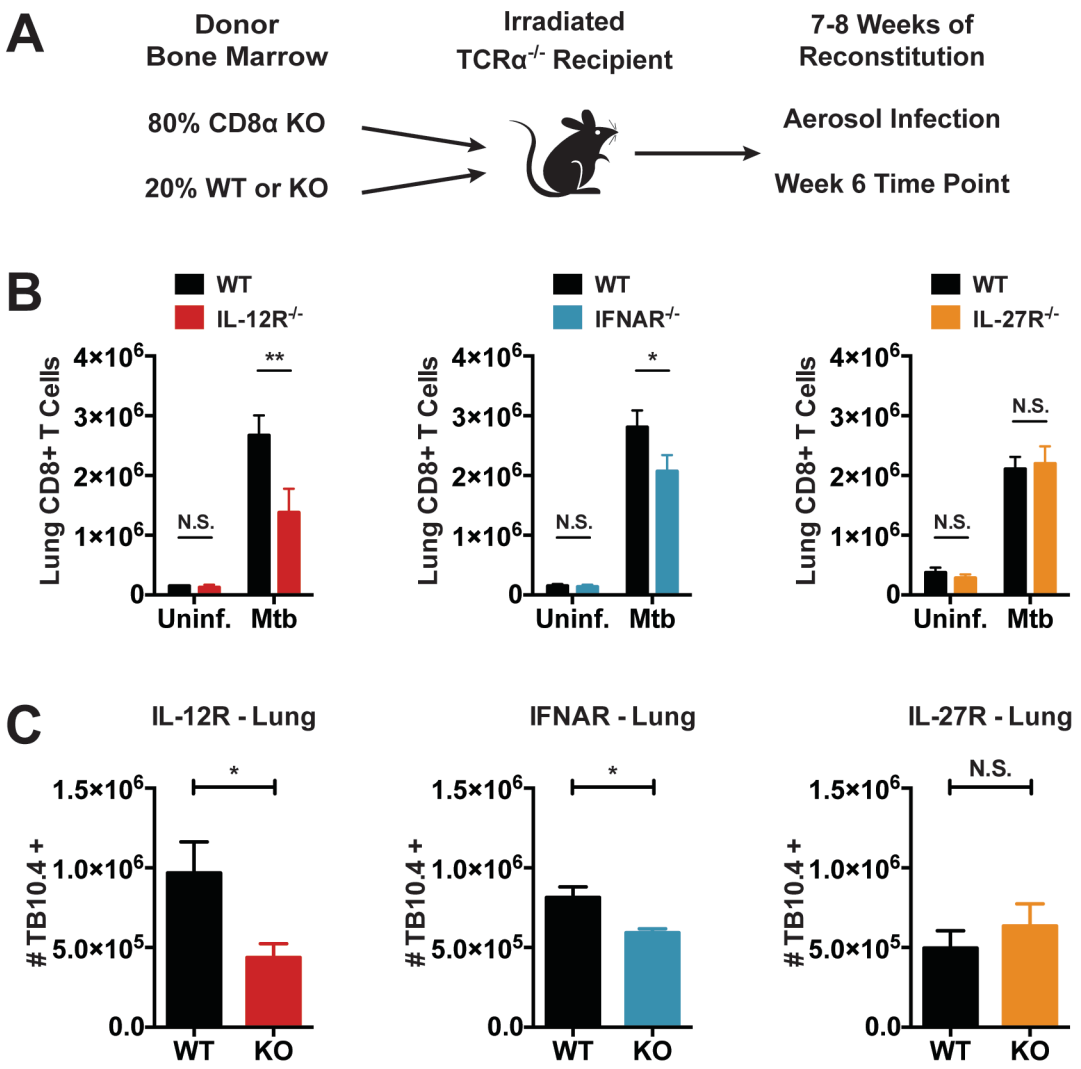

Figure 2-9. The "4:1" mixed bone marrow chimera system: A different approach to studying the role of IL-12R, IFNAR, and IL-27R on CD8$^+$ T cells.

(A) Overview of 4:1 mixed bone marrow chimera generation. Briefly, TCRα$^{-/-}$ mice were irradiated and reconstituted with a mixture of donor cells consisting of 80% CD8α$^{-/-}$ bone marrow and 20% of either WT or receptor knockout bone marrow (IL-12R$^{-/-}$, IFNAR$^{-/-}$, or IL-27R$^{-/-}$). (B) Total number CD8$^+$ T cells in the lungs of uninfected mice and mice 6 weeks post-infection. (C) Total number CD8$^+$ TB10.4$^+$ T cells in the lungs of mice 6 weeks post-infection. (n = 4-5 mice per group). *P < 0.05, **P < 0.01 (unpaired Student's t-test). Data are representative of two independent experiments.

Mtb (*Fig 2-9B and data not shown*). After six weeks of infection, the chimeras with IL-12R$^{-/-}$, IFNAR$^{-/-}$, or IL-27R$^{-/-}$ CD8^{+} T cells had the same bacterial burden as control chimeras with WT CD8^{+} T cells and all chimeras survived (data not shown).

As a model, the 4:1 chimeras recapitulated many of the phenotypes observed in the 1:1 mixed bone marrow chimeras, and the loss of IL-12 signaling again had the largest effect on CD8^{+} T cell expansion in the lungs following infection (*Figure 2-9B and 2-9C*). Compared to the 1:1 mixed bone marrow chimeras, this defect in IL-12R$^{-/-}$ CD8^{+} T cell expansion was less dramatic. In the 4:1 chimera model, type 1 IFN signaling also had a less dramatic effect on CD8^{+} T cell expansion, and the loss of IL-27R did not impact CD8^{+} T cell numbers during infection. This underscores the value of the 1:1 mixed bone marrow chimera model where WT and KO cells can be directly compared in the same host environment. It is possible that the effect of type 1 IFN and IL-27 on CD8^{+} T cell expansion is only evident when KO cells are in direct competition with the WT response. Overall, this data supports the observation that type 1 IFN and IL-27 play a smaller role in supporting expansion. Beyond expansion, CD8^{+} T cell differentiation and function were also affected in the 4:1 chimera system. Again, only IL-12 and IL-27 influenced the differentiation of effector cells (*Figure 2-10*). IL-12 was required for efficient IFN-γ production (*Figure 2-11A*), and granzyme B levels were reduced in IL-12R$^{-/-}$, IFNAR$^{-/-}$, and IL-27R$^{-/-}$ CD8^{+} T cells (*Figure 2-11B*).

An *in vivo* cytotoxicity assay was performed with the 4:1 chimeras by assessing the specific killing of fluorescently labeled target splenocytes loaded with TB10.4$_{4-11}$. We observed the killing of target cells in the both the lungs and spleens of infected mice, and only IL-12R$^{-/-}$ CD8^{+} T cells demonstrated diminished specific killing

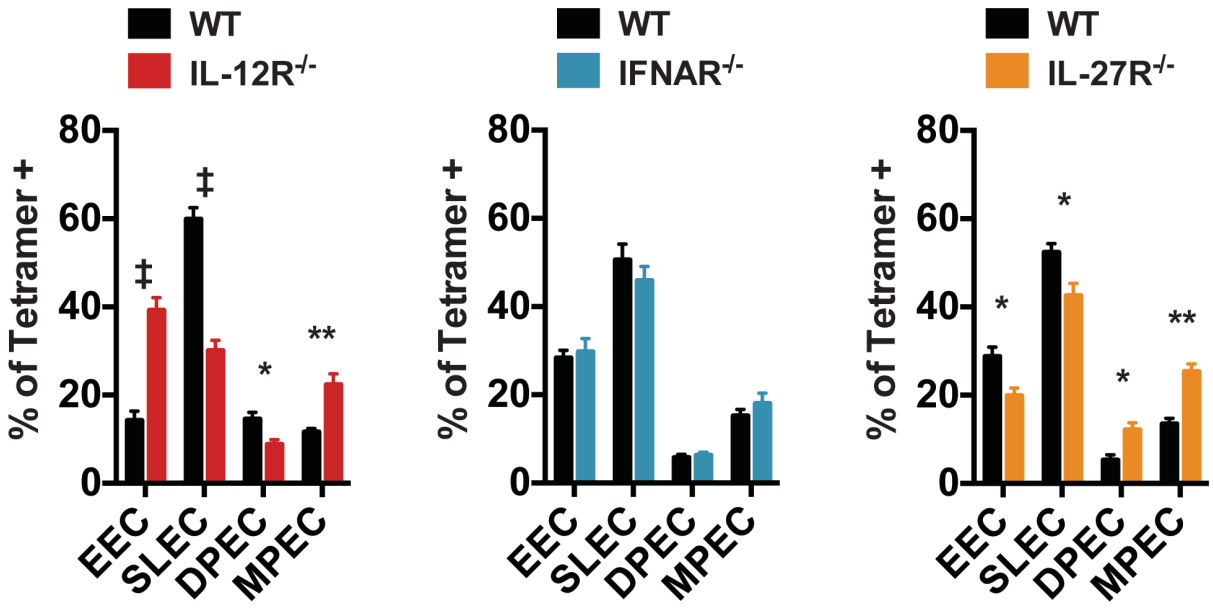

Figure 2-10. IL-12 and IL-27 influence effector CD8$^+$ T cells differentiation in the 4:1 chimera model.

Phenotypic analysis of CD8$^+$ TB10.4$^+$ cells based on KLRG1 and CD127 staining (EEC – Early Effector Cells; SLEC – Short-Lived Effector Cells; DPEC – Double Positive Effector Cell; MPEC – Memory Precursor Effector Cell) Each bar represents the mean ± SEM (n = 4-5 mice per group) *P < 0.05, **P < 0.01, ***P < 0.001, ‡P < 0.0001 (unpaired Student's t-test). Data are representative of two independent experiments.

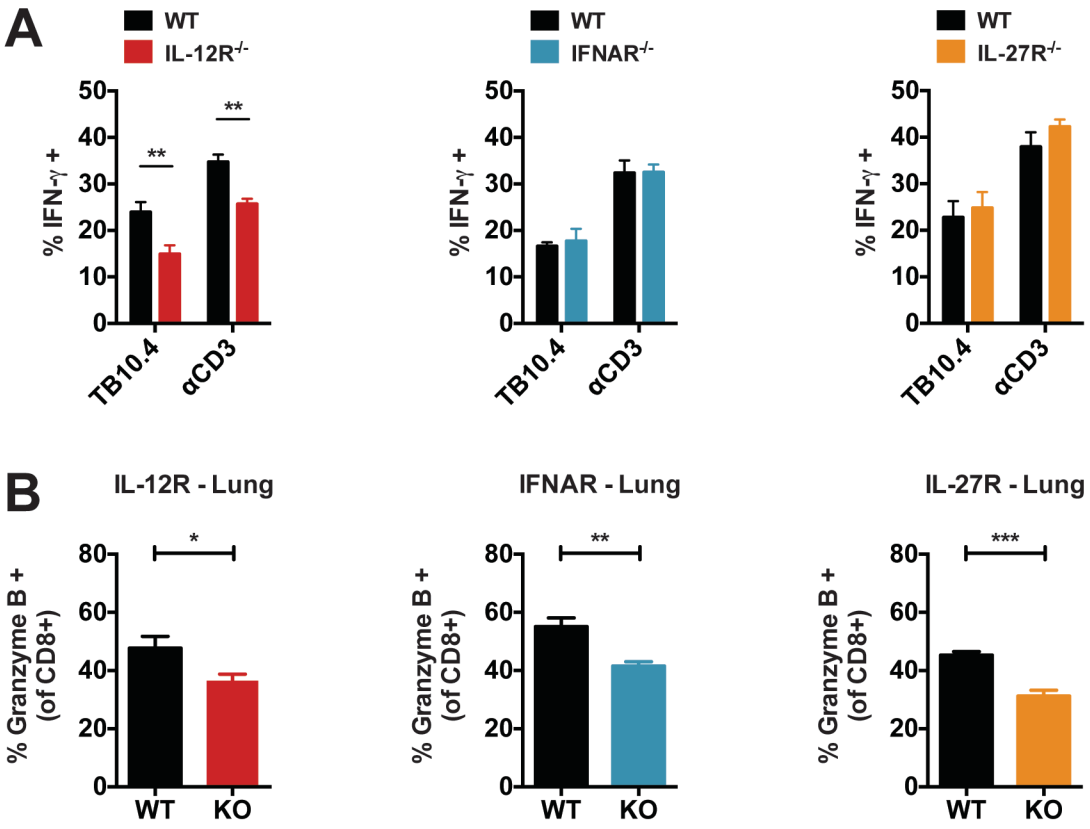

Figure 2-11. Only IL-12 is required for IFN-γ production, but IL-12, type 1 IFN, and IL-27 all enhance granzyme B levels.

(A) The percentage of WT and KO $CD8^+$ T cells positive for IFN-γ as determined by ICS. Lung cells from 4:1 chimeras were stimulated ex vivo with either TB10.4 peptide or αCD3 and αCD28. (B) The percentage of $CD8^+$ T cells positive for intracellular granzyme B staining in the lung. Each bar represents the mean ± SEM (n = 4-5 mice per group) *P < 0.05, **P < 0.01 (unpaired Student's t-test). Data are representative of two independent experiments.

(*Figure 2-12A and 2-12B*). Given that IL-12R$^{-/-}$ CD8^{+} T cells have a profound defect in the expansion of antigen-specific cells (*Figure 2-9B and 2-9C*), it was important to determine if the reduced level of specific killing resulted from fewer antigen-specific cells or actually reflected the functional capacity of the cytotoxic lymphocytes. By plotting the absolute number of TB10.4-specifc CD8^{+} cells in the spleen versus specific killing, it was apparent that cell number had the greatest impact on the percentage of target cells lysed (*Figure 2-13*). This correlation was most robust in the spleens where a higher number of target cells were recovered, but similar results were observed in the lungs (data not shown). These findings suggest that cytolytic cells are still generated in the absence of IL-12, type 1 IFN, or IL-27 signaling, despite an overall reduction in granzyme B levels. Multiple molecules and several different pathways contribute to cytolysis (117,118), thus it is likely that the loss of individual cytokine receptors does not have a substantial impact this effector function.

IL-12 mediates CD8^{+} T cell priming in the lung draining lymph node, while type 1 IFN and IL-27 exert the majority of their effects in the lungs.

IL-12, type 1 IFN, or a combination of the two are known to provide a necessary third signal for priming CD8^{+} T cells in multiple infections, but their role as “signal 3” cytokines during tuberculosis is unknown. At present, IL-27 is not considered a signal 3 cytokine, but it has several of the same attributes. IL-27 can support CD8^{+} T cell expansion, the induction of cytolytic activity, and it influences CD8^{+} T cell differentiation. Because IL-12, type 1 IFN, and IL-27 all influence the magnitude of the CD8^{+} T cell response during tuberculosis, we examined if these effects are mediated at the onset of

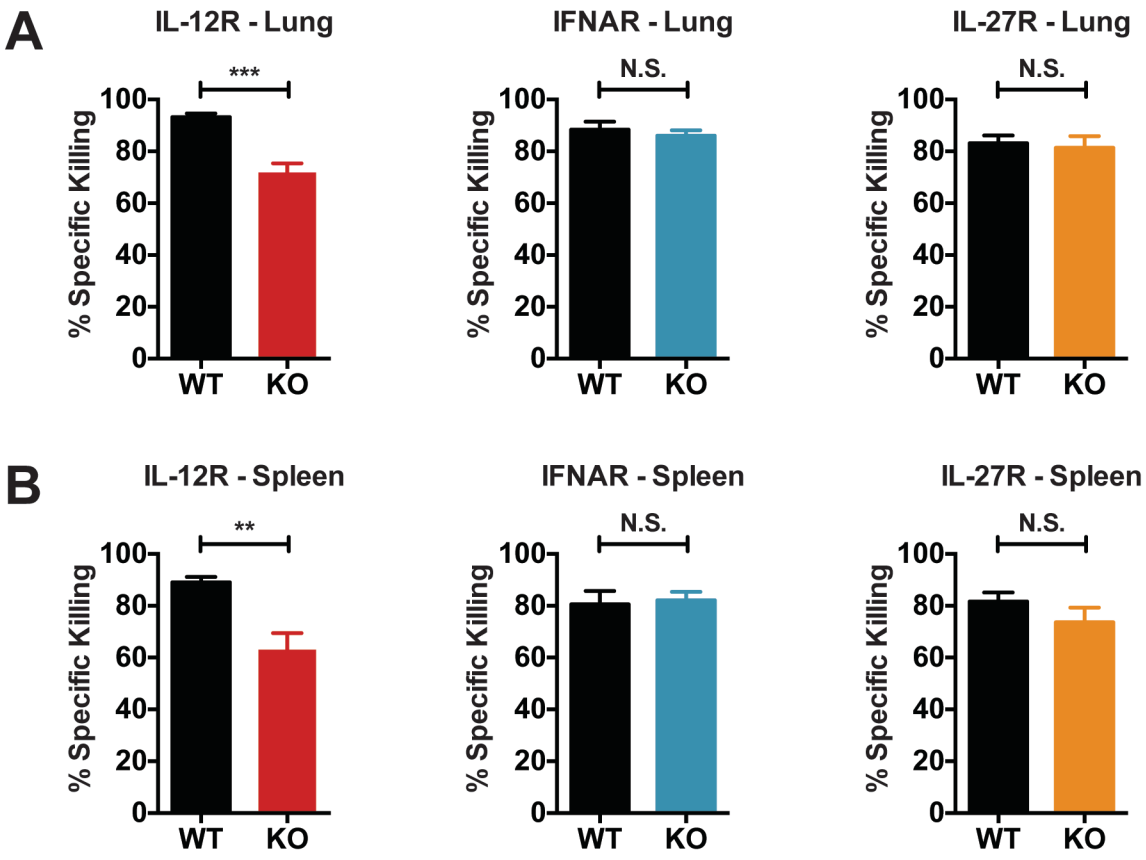

Figure 2-12. IL-12R$^{-/-}$ CD8^{+} T cells exhibit reduced specific killing in an *in vivo* cytotoxicity assay.

In vivo cytolytic activity of TB10.4-specific CD8^{+} T cells in the lungs (A) and spleens (B) of infected 4:1 chimeras. Here, target cells were pulsed with 100 nM of TB10.4 peptide. Specific killing was calculated as described in the Materials and Methods. Each bar represents the mean ± SEM (n = 4-5 mice per group) N.S. = Not Significant, *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Student's t-test). Data are representative of two independent experiments.

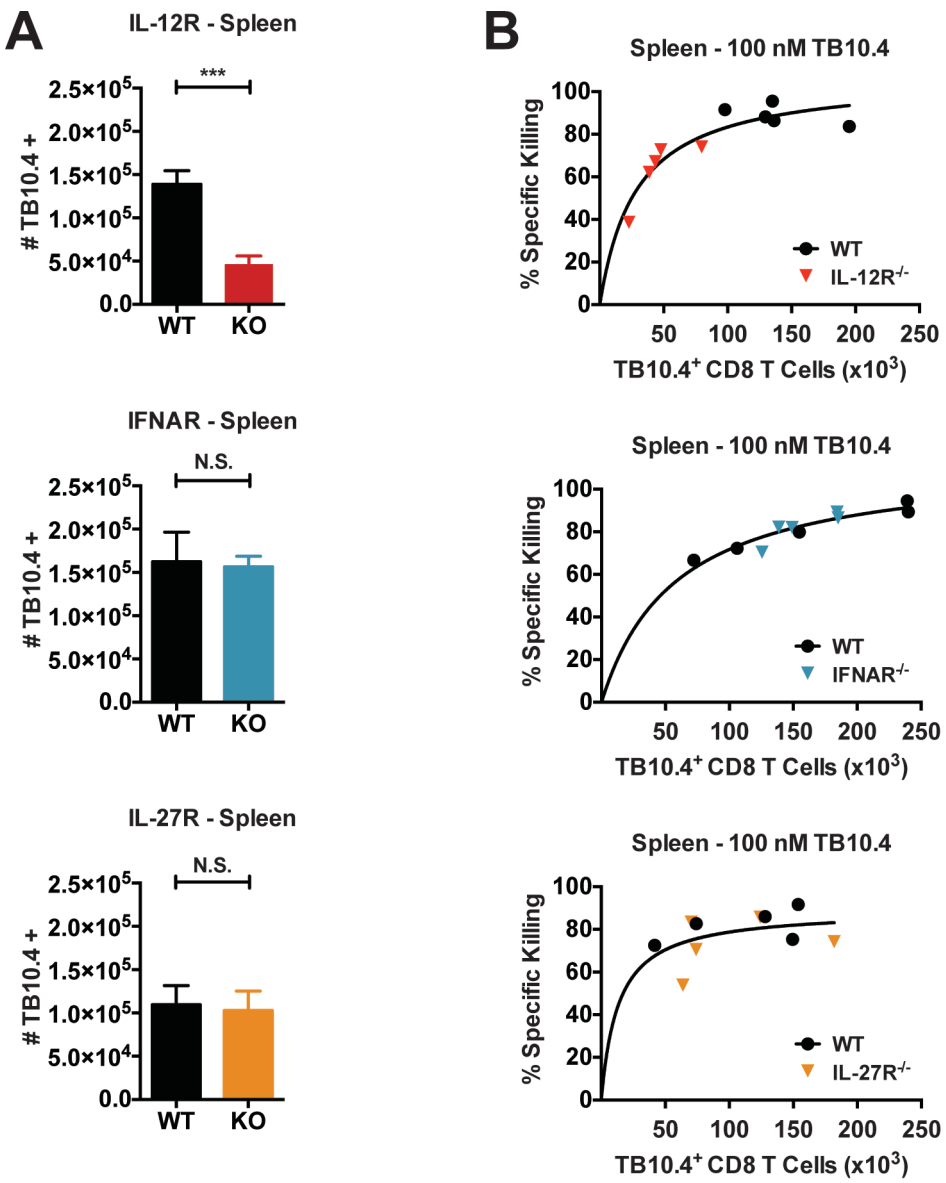

Figure 2-13. The reduced specific killing by IL-12R$^{-/-}$ CD8^{+} T cells is the result of diminished CD8^{+} T cell numbers.

(A) The total number CD8^{+} TB10.4^{+} T cells in the spleens of mice 6 weeks post-infection. (B) Nonlinear regression analysis of TB10.4-specific CD8+ T cell number vs. specific killing for each group of 4:1 chimeras. In every experiment, the data is best fit by a single curve. Each bar represents the mean ± SEM (n = 4-5 mice per group) *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Student's t-test). Data are representative of two independent experiments.

priming or occur later in the lungs.

To address this question, we utilized a retrogenic mouse model in which a high percentage of $CD8^+$ T cells are specific for the immunodominant antigen $TB10.4_{4\text{-}11}$. One of the key advantages of retrogenic mice is that naïve antigen-specific $CD8^+$ T cells can be generated on nearly any genetic background, thus providing a source of naïve $TB10.4_{4\text{-}11}$-specific IL-$12R^{-/-}$, $IFNAR^{-/-}$, and IL-$27R^{-/-}$ $CD8^+$ T cells. Our lab has recently validated this system as a means of obtaining large numbers of functional $TB10.4_{4\text{-}11}$-specific $CD8^+$ T cells, and upon adoptive transfer, these cells are primed in the draining lymph node approximately 11 days following aerosol infection (Nunes-Alves et al., *in press*). This delay in T cell priming results from the delayed transfer of Mtb from the lung to the draining lymph node (119). In these priming experiments, equal numbers of congenically marked WT and receptor KO retrogenic (Rg) $TB10.4_{4\text{-}11}$-specific $CD8^+$ T cells were transferred into recipient mice 7 days after low-dose aerosol infection, and the ratio of WT and KO Rg $CD8^+$ T cells was assessed at days 8, 11, 13, and 15.

At day 8, the ratio of WT and receptor KO Rg $CD8^+$ T cells was unaltered from the ratio of cells injected at day 7 and no group of cells diluted proliferation dye (*Figure 2-14A* and data not shown). By day 11 in the mediastinal lymph node, IL-$12R^{-/-}$ Rg $CD8^+$ T cells were underrepresented relative to WT cells and continued to lag behind through days 13 and 15 (*Figure 2-14A*). Throughout the experiment, $IFNAR^{-/-}$ and IL-$27R^{-/-}$ Rg $CD8^+$ T cells maintained a consistent ratio with WT cells in the lymph node, indicating that type 1 IFN and IL-27 are dispensable for $CD8^+$ T cell priming following Mtb infection (*Figure 2-14A*). Overall, the expansion of $IFNAR^{-/-}$ and IL-$27R^{-/-}$ $CD8^+$ T cells was equivalent to WT cells in the lymph node at all the time points (*Figure 2-14B*).

Figure 2-14. IL-12 acts as a Signal 3 cytokine during tuberculosis and promotes CD8$^+$ T cell priming in the lung draining lymph node.

Equal numbers of retrogenic (Rg) TB10.4-specific CD8$^+$ T cells were transferred into mice 7 days after low-dose aerosol infection with Mtb. (A) The percentage of total retrogenic (Rg) cells that were WT or KO for the indicated cytokine receptor in the mediastinal lymph node at days 8, 11, 13 and 15 following infection. (B) Total number of WT and KO Rg cells detected in the lymph node at the indicated time points. (C) Histograms depicting the dilution of the proliferation dye efluor 450 in Rg cells in lymph node at day 11. Each group of samples (WT or KO) was concatenated into a single histogram. Each bar or point represents the mean ± SEM (n = 4-5 mice per group) ‡P < 0.0001 (Holm-Šídák multiple comparisons testing following two-way ANOVA). Data are representative of two independent experiments.

Figure panels on following page.

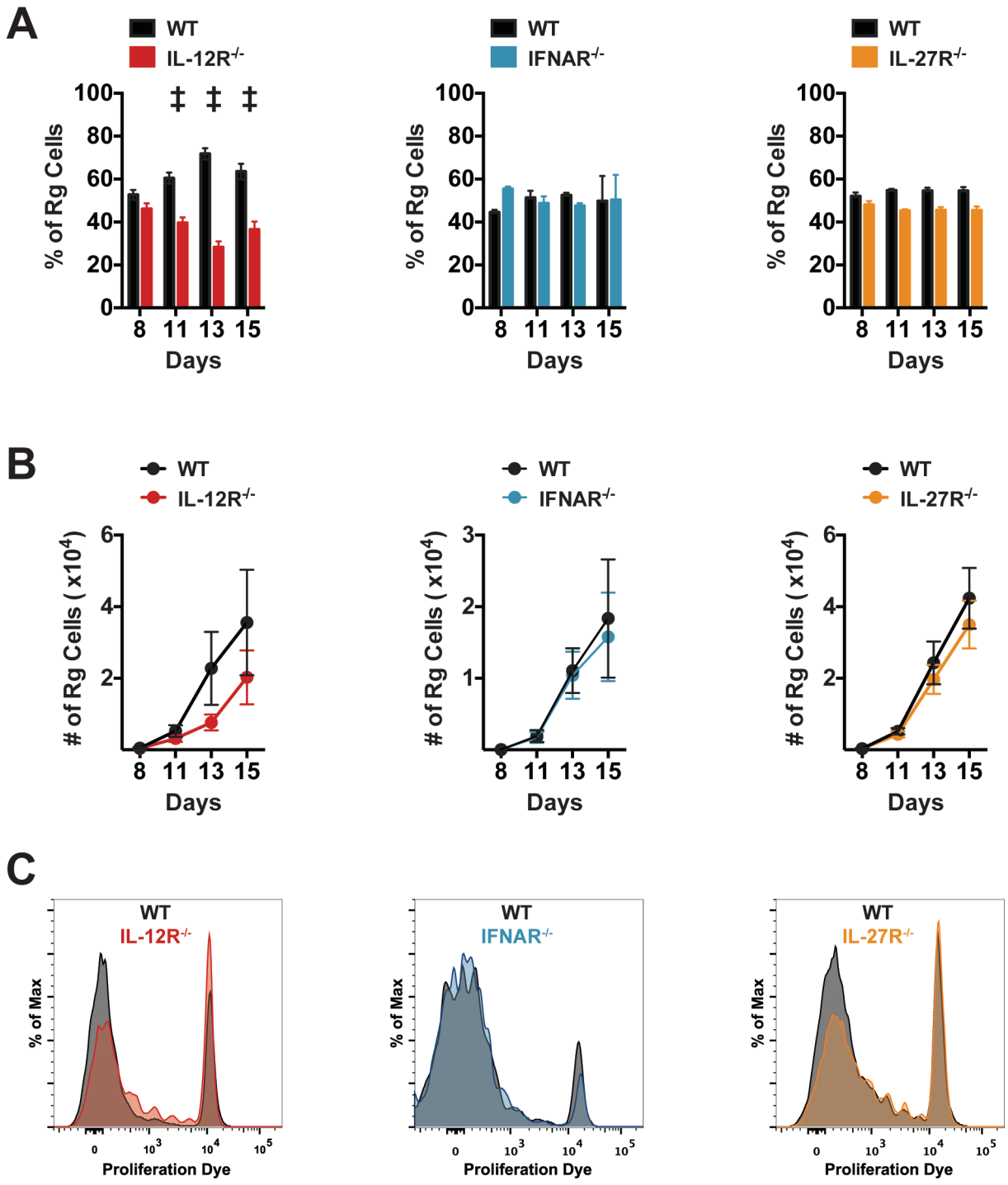

Figure 2-14 (Continued). IL-12 acts as a Signal 3 cytokine during tuberculosis and promotes CD8$^+$ T cell priming in the lung draining lymph node.

Surprisingly, IL-12R$^{-/-}$ Rg CD8^{+} T cells still expanded significantly in the lymph node, though they did lag behind WT cells (*Figure 2-14B*). Indeed, IL-12R$^{-/-}$ Rg CD8^{+} T cells in the lymph node substantially diluted their proliferation dye by day 11 post-transfer, though not as efficiently as WT cells (*Figure 2-14C*). These observations indicate that additional signals present in the lymph node facilitate CD8^{+} T cell priming and suggest that an additional signal 3 cytokine may exist. Additionally, type 1 IFN or IL-27 may be able to support priming in the absence of IL-12. IFNAR$^{-/-}$ and IL-27R$^{-/-}$ Rg CD8^{+} T cells diluted their proliferation dye equivalent to WT cells by day 11, reinforcing that these signals are not needed to prime cells in the lymph node when IL-12 is present (*Figure 2-14C*).

In the same priming experiments, the expansion of the transferred cells was monitored in the lungs of recipient mice. Similar to the observations in the lymph node, IL-12R$^{-/-}$ Rg CD8^{+} T cells continued to expand over the course of the experiment; however, they underperformed relative to WT cells (*Figure 2-15A and 2-15B*). This suggests that IL-12 is necessary for the efficient priming of CD8^{+} T cells in the lymph as well as their continued expansion in the lungs. Though type 1 IFN and IL-27 are dispensable for CD8^{+} T cell priming, their influence on CD8^{+} T cell expansion in the lungs was observable at 13 days post infection, and the percentage of IFNAR$^{-/-}$ and IL-27R$^{-/-}$ Rg CD8^{+} T cells decreased relative to their WT counterparts at days 13 and 15 (*Figure 2-15A and 2-15B*). These data suggest that type 1 IFN and IL-27 primarily influence the accumulation of CD8^{+} T cells in the periphery after priming has occurred.

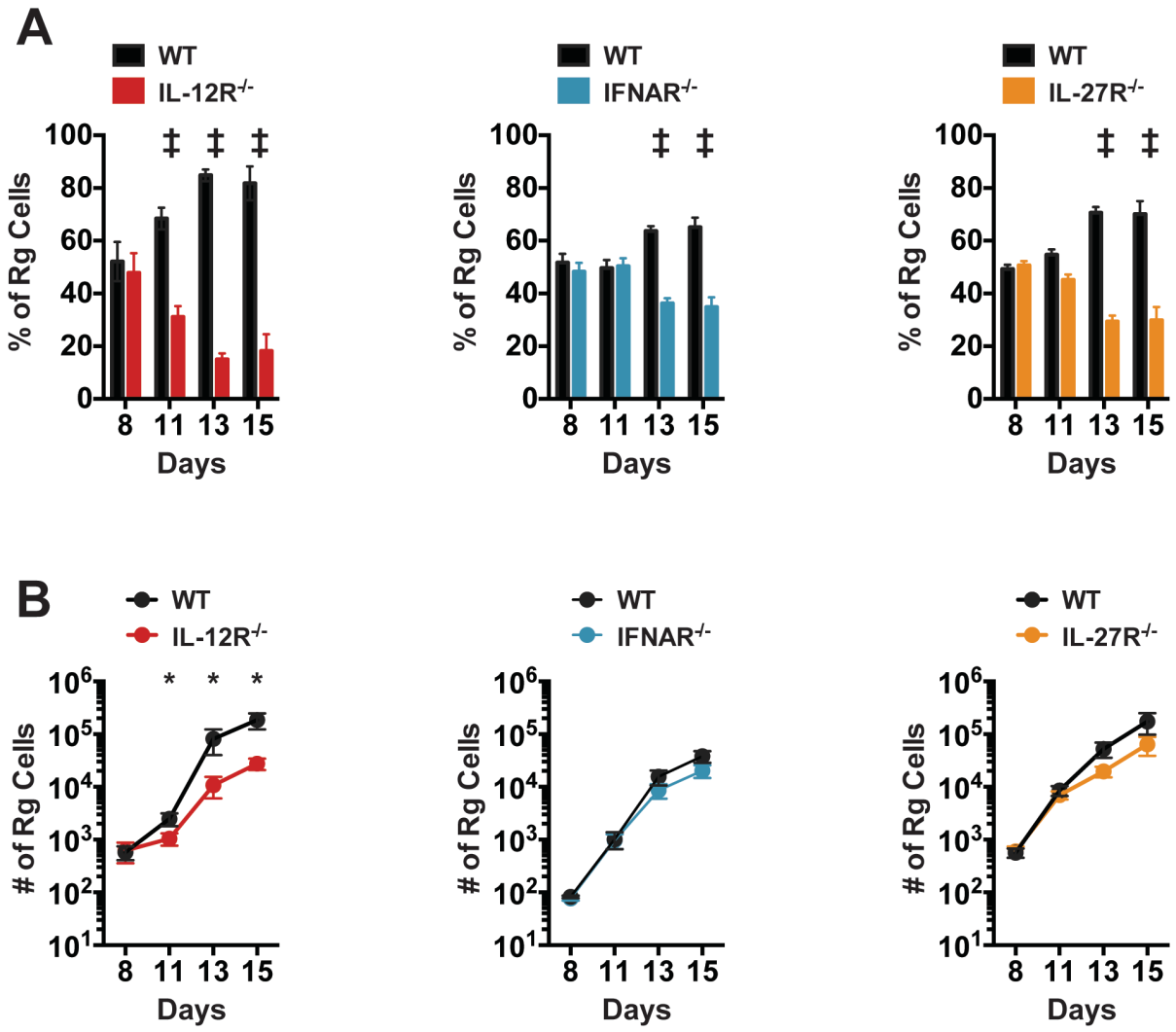

Figure 2-15. IL-12, type 1 IFN, and IL-27 augment the expansion of CD8$^+$ T cells in the lungs during tuberculosis.

Equal numbers of retrogenic (Rg) TB10.4-specific CD8$^+$ T cells were transferred into mice 7 days after low-dose aerosol infection with Mtb. (A) The percentage of total retrogenic (Rg) cells that were WT or KO for the indicated cytokine receptor in the lungs at days 8, 11, 13 and 15 following infection. (B) Total number of WT and KO Rg cells detected in the lungs at the indicated time points. Each bar or point represents the mean ± SEM (n = 4-5 mice per group) ‡P < 0.0001 (Holm-Šídák multiple comparisons testing following two-way ANOVA). Data are representative of two independent experiments.

Discussion

Infection with Mtb elicits a complex inflammatory milieu that shapes the adaptive immune response. Here, we examined the impact of inflammation on $CD8^+$ T cell function by focusing on three cytokines known to influence host resistance. Using 1:1 mixed bone marrow chimeras and the transfer of naïve Ag-specific $CD8^+$ T cells, we show that IL-12 is a major regulator of the $CD8^+$ T cell response during tuberculosis. Following aerosol infection, IL-12 is essential for efficient $CD8^+$ T cell priming in the lymph node and subsequent expansion in the lungs. IL-12 also promotes the terminal differentiation of SLECs and enhances IFN-γ production. For these reasons, IL-12 is the dominant signal 3 cytokine during tuberculosis.

However, IL-12 is not acting alone in the infected host. We demonstrate a supporting role for both type 1 IFN and IL-27 in expansion. These effects are not observed in the lymph node during the priming of naïve $CD8^+$ T cells and only become evident in the infected lungs. Because they are dispensable for priming, we argue type 1 IFN and IL-27 are not acting as signal 3 cytokines. Comparatively, IL-12 has the most dramatic impact on $CD8^+$ T cell numbers, likely because it influences expansion in both the lymph node and the lungs. Though less pronounced, type 1 IFN and IL-27 each have a non-redundant role augmenting the magnitude of the $CD8^+$ T cell response in the lungs. This is most evident in the 1:1 mixed bone marrow chimeras, where WT and KO cells are in direct competition within the same inflammatory environment. This complex involvement of multiple inflammatory cytokines is similar to other infections, where IL-12 and type 1 IFN both support $CD8^+$ T cell expansion (98). In this way, $CD8^+$

T cells reflect the inflammatory environment of the host, responding in different degrees to each cytokine present.

Type 1 IFN negatively impacts host immunity to Mtb through a number of mechanisms, but a direct effect on T cells has not been examined. In 1:1 mixed bone marrow chimeras, type 1 IFN supported $CD8^+$ T cell expansion and had no impact on $CD4^+$ T cell numbers (data not shown). Based on these findings, type 1 IFN does not have a directly negative effect on T cells and instead boosts the expansion of $CD8^+$ T cells in the lungs. Beyond this effect, type 1 IFN was not required for $CD8^+$ T cell differentiation or function in our experiments.

We were surprised to discover IL-27 had no impact on effector functions. IL-27 is a critical promoter of IFN-γ production by $CD8^+$ T cells in other infections (58); but we did not observe this during tuberculosis. It is possible that the strong effect of IL-12 masks a role for IL-27; however, this seems unlikely. During *T. gondii* infection, IL-27 is necessary for IFN-γ production, while IL-12 levels drive the generation of effector $CD8^+$ T cells (58,99,120). Thus in other infections, IL-27 is capable of driving IFN-γ-production even in the presence of high IL-12. The effects of IL-27 on $CD8^+$ T cell-derived IFN-γ are likely pathogen-specific, and it is clearly unnecessary during tuberculosis. The loss of IL-27 signaling does influence $CD8^+$ T cell differentiation. It is dispensable for SLEC generation, but limits CD127 expression. Thus, IL-$27R^{-/-}$ cells were more likely to become DPECs and MPECs. Based on this observation, it is tempting to speculate that IL-27 limits the formation of memory during tuberculosis; however, such a possibility cannot be addressed with our current data.

IL-12, type 1 IFN, and IL-27 all promote granzyme B production following Mtb infection, but no loss in *in vivo* cytolytic activity is observed. Mice with IL-12R$^{-/-}$ CD8^{+} T cells show reduced specific killing; however, out data indicate this results from reduced CD8^{+} T cell numbers not cytolytic function. In fact, IL-12R$^{-/-}$ CD8^{+} T cells were highly capable of lysing targets cells *in vivo*. This observation is likely due to redundancy in regulating cytolysis. For example, multiple cytokines may support cytolytic activity, therefore, the loss of a single cytokine fails to perturb specific killing. Additionally, there are multiple molecules and mechanisms involved in cytolysis during tuberculosis (117,118), thus a reduction in granzyme B levels alone may have a marginal impact overall. In particular, perforin is critical for cytolysis during tuberculosis (117). However, a reliable method for perforin staining in murine cells has only recently become available (121), thus we did not assess perforin expression in these experiments. Nonetheless, cells retain a high degree of cytolytic activity even in the absence of IL-12. This suggests that cytolysis is a robust effector mechanism found even in dysfunctional cells, such as IL-12R$^{-/-}$ CD8^{+} T cells.

Using adoptively transferred retrogenic cells, we tracked CD8^{+} T cell priming in the lung draining lymph. IL-12 was critical for supporting priming, however, naïve IL-12R$^{-/-}$ CD8^{+} T cells still expanded in all of our experiments. This implies other signals support CD8^{+} T cell priming in the absence of IL-12. During tuberculosis, IL-23 can compensate for the absence of IL-12 to promote CD4^{+} T cell responses. We used IL-12Rβ2$^{-/-}$ cells in all our experiments, thus IL-23 signaling remained intact. It is possible that IL-23 supported CD8^{+} T cell expansion; however, *in vitro* experiments have failed to associate IL-23 with signal 3 activity, making it an unlikely candidate (100). Though type

1 IFN and IL-27 are dispensable for priming, it is possible they promote it in the absence of IL-12. We have recently acquired IL-12R/IFNAR double knockout mice and will soon test the priming ability of these cells. During vaccinia virus infection, neither IL-12 nor type 1 IFN are required to generate a $CD8^+$ T cell response, thus unknown sources of signal 3 must exist. IL-21 is a potential candidate, and it acts as a signal 3 cytokine *in vitro* (discussed in Chapter 3) (122). Such a scenario would be somewhat unconventional, because activated $CD4^+$ T cells produce IL-21, not DCs.

Our current understanding of $CD8^+$ T cell responses during tuberculosis is limited. These data illuminate a portion of the cytokine network regulating these cells and illustrate the complex ways in which inflammation shapes the adaptive immune response. $CD8^+$ T cells are similar to $CD4^+$ T cells in their requirement for IL-12, but have the opposite response to IL-27 signaling. It is intriguing to consider that IL-27 functions to limit a pathological $CD4^+$ T cell response while simultaneously supporting the expansion of $CD8^+$ T cells. In this way, IL-27 can possibly achieve a balance of IFN-γ-producing cells in the lungs. Relationships such as these must be understood if we hope to design better vaccination strategies in the future.

Materials and methods

Mice

C57BL/6 (WT), CD45.1 (B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ), CD90.1 (B6.PL-Thy1^{a}/CyJ), TCR$\alpha^{-/-}$ (B6.129S2-Tcra^{tm1Mom}/J), CD8$\alpha^{-/-}$ (B6.129S2-Cd8a^{tm1Mak}/J), IL-12 receptor beta 2 deficient (IL-12R$^{-/-}$: B6.129S1-Il12rb2^{tm1Jm}/J), and IL-27 receptor alpha deficient (IL-27R$^{-/-}$: B6N.129P2-Il27ra^{tm1Mak}/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Interferon-α/β receptor deficient mice (IFNAR$^{-/-}$) were obtained from Dr. Raymond M. Welsh and were previously described (123,124). Mice were 6 to 10 weeks old at the start of all experiments. All animal experiments were performed in accordance with National and European Commission guidelines for the care and handling of laboratory animals and were approved by the National Veterinary Directorate and by the local Animal Ethical Committee or Institutional Animal Care and Use Committee (Animal Welfare Assurance no. A3023-01 [DFCI] or A3306-01 [UMMS]), under Public Health Service assurance of Office of Laboratory Animal Welfare guidelines). Mice infected with *M. tuberculosis* were housed in a biosafety level 3 facility under specific pathogen-free conditions at DFCI or at UMMS.

Generation of mouse bone marrow chimeras

1:1 mixed bone barrow chimeras were made by lethally irradiating CD90.1^{+} recipients (2 doses of 600 rads separated by three hours). Bone marrow was flushed from the femurs, tibia, and humeri of donor mice and RBC lysed. Bone marrow cells were then enumerated and groups were combined in a 1:1 ratio. Each recipient mouse

received a total of 10^7 bone marrow cells (5×10^6 of WT and 5×10^6 of KO) via lateral tail vein injection and was kept on antibiotic-treated water for 5 weeks following irradiation. Mice were checked for reconstitution by retro-orbital bleeding to assess the ratio of donor cells in the peripheral blood by flow cytometry. Bone marrow chimeras were infected with Mtb 8-10 weeks after transfer of the bone marrow cells. For 4:1 chimeras, TCR$\alpha^{-/-}$ mice were given a lower dose of radiation (2 doses of 500 rads separated by three hours). The ratio of donor cells in these chimeras was 80% CD8$\alpha^{-/-}$ and 20% of the indicate KO bone marrow.

Generation of retrogenic mice

TCR retroviral constructs were generated as 2A-linked single open reading frames using PCR and cloned into a murine stem cell virus-based retroviral vector with a GFP marker as previously described (125). Details of cloning strategies and primer sequences are available upon request (Samuel.behar@umassmed.edu). Retroviral-mediated stem cell gene transfer was performed as previously described (125).

Experimental infection and bacterial quantification

Infection with *M. tuberculosis* (Erdman strain) was performed via the aerosol route, and mice received a day 1 inoculum of 50-200 CFU. A bacterial aliquot was thawed, sonicated twice for 10 seconds in a cup horn sonicator, and then diluted in 0.9% NaCl–0.02% Tween 80. A 15 ml suspension of *M. tuberculosis* was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technologies) and mice were infected using a nose-only exposure unit (Intox Products). Alternatively, the bacterial aliquot was

diluted in a final volume of 5ml, and mice were infected using a Glas-Col aerosol-generation device. At different times post-infection, mice were euthanized by carbon dioxide inhalation, organs were aseptically removed, individually homogenized and viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H11 agar plates. Plates were incubated at 37ºC and *M. tuberculosis* colonies were counted after 21 days.

FACS analysis

Cell suspensions from lung, spleen and lymph nodes were prepared by gentle disruption of the organs through a 70µm nylon strainer (Fisher) or using the GentleMacs apparatus (Miltenyi Biotec, Germany) according to the manufacturer's instructions. For lung preparations, tissue was digested for 30-60 min at 37°C in cRPMI with 300U/ml collagenase (Sigma) prior to straining. Erythrocytes were lysed using a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM sodium EDTA pH 7.2) and, after washing, cells were resuspended in supplemented RPMI (cRPMI - 10% heat inactivated FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml streptomycin and 50 U/ml penicillin, all from Invitrogen) or MACS buffer (Miltenyi Biotec, Germany). Cells were enumerated in 4% trypan blue on a hemocytometer or using a MACSQuant flow cytometer (Miltenyi Biotec, Germany). Surface staining was performed with antibodies specific for mouse CD3 (clone 17A2), CD3ε (clone 145-2C11), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD19 (clone 6D5), CD44 (clone IM7), CD62L (clone MEL-14), CD45.1 (clone A20), CD45.2 (clone 104), CD90.1 (clone OX-7), CD90.2 (clone 53-2.1), CD127 (clone A7R34), KLRG1 (clone 2F1/KLRG1) and Va2

(clone B20.1), (from Biolegend, CA, USA, or from BD Pharmingen, CA, USA). The tetramers of TB10.$4_{4–11}$-loaded H-2 K^b were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA, USA). All staining was performed for 20 min at 4°C and, unless otherwise stated, cells were fixed before acquisition with 1% formaldehyde in PBS for 30-60 min. Cell analysis was performed on a FACS Canto (Becton Dickinson, NJ, USA) or on a MACSQuant flow cytometer (Miltenyi Biotec, Germany). Data were analyzed using FlowJo Software (Tree Star, OR, USA). For FACS analysis, single-lymphocyte events were gated by forward scatter versus height and side scatter for size and granularity.

Intracellular cytokine staining

5×10^5-1×10^6 cells were plated in each well of a round bottom 96-well plate and incubated in the presence of TB10.4_{4-11} peptide (10 μM; New England Peptide). Incubation in the presence of αCD3/αCD28 (1 μg/mL; BioLegend) or in the absence of stimuli were used as positive and negative controls, respectively. Cells were incubated for 1 hour at 37ºC, at which point GolgiPlug solution (BD Pharmingen, CA, USA) was added to each well for the remaining 4 hours. Cells were collected after the 5 hours stimulation and then surface stained with the antibodies described above, followed by intracellular staining for IFN-γ (clone XMG1.2), TNF (clone MPX6-T22), or granzyme B (clone gb11) using the BD Permwash Kit (BD Pharmingen, CA, USA) as per manufacturer's instructions.

In vivo cytotoxicity assay

In vivo cytotoxicity was determined using peptide-coated splenocytes differentially labeled with the fluorescent dyes CFSE and efluor 450 (eBiosciences) as previously described (12, 31)(117). All target cells were obtained from uninfected mice. Target cells were labeled in PBS for either 20 minutes (efluor 450) or 10 minutes (CFSE) at room temperature, followed by extensive washing. Target cell populations were pulsed with 10-0.01 μM of the TB10.4_{4-11} peptide at 37°C for 1 hour in complete medium or left unpulsed. Labeled populations were mixed at an equal cell ratio and injected IV into age-matched uninfected and infected recipient mice ($2.5x10^6$ of each labeled population per mouse). After 20 hours, recipient spleens and lungs were harvested and single-cell suspensions were made as described. Ratios of recovered CFSE- and efluor 450-labeled target lymphocyte populations were determined by flow cytometry. Percent specific killing was determined by the following formula: percent specific killing = 100 – (100 - (ratio in infected mice)/(ratio in uninfected mice)), where ratio = percent peptide-pulsed target cells/percent unpulsed target cells.

Adoptive T cell transfer

Single cell suspensions of pools of spleens and lymph nodes from naive retrogenic mice (6 to 12 weeks post reconstitution) were prepared. $CD8^+$ T cells were purified from each suspension using the $CD8^+$ T cell isolation kit and magnetic separation (STEMCELL Technologies Inc, Canada). After purification, cells were counted and transferred via the tail vein into congenically marked recipients (CD45.1 or CD90.1), which had been infected 7 days earlier with virulent Mtb (Erdman) via the aerosol route. For all experiments, $3×10^4$ -$5×10^4$ cells of each group were transferred

into each recipient.

Measurement of cell proliferation

For analysis of cell proliferation of retrogenic cells after adoptive transfer, bead-purified naïve Rg cells (see above) were labeled with 5 μM cell proliferation dye efluor 450 (eBiosciences) in PBS for 20 min at room temperature, followed by extensive washing.

Cell isolation and microarray analysis

Female C57BL/6 mice were infected with Mtb Erdman as described above. At the indicated time points, mice were euthanized by cervical dislocation and lungs were harvested after perfusion with collagenase containing media. Lungs were allowed to digest in collagenase-containing media for 15 minutes before being homogenized into single cell suspensions. At this point, the lungs from 3 individual mice were combined into a single sample. T cells were then purified by negative magnetic bead selection (Miltenyi Biotec, Germany). Purified cells were stained to distinguish $CD4^+$ and $CD8^+$ T cells (CD19, CD3, CD4, CD8). For cell sorting, stained cells were suspended in MACS buffer (Miltenyi Biotec, Germany) and deposited in collection tubes using a BD Canto flow cytometer (Becton Dickinson, NJ, USA). 50,000 $CD19^-CD3^+CD4^+$ or $CD19^-CD3^+CD8^+$ cells were sorted directly into TRIzol Reagent (Life Technologies, California) and immediately frozen. RNA extraction, microarray hybridization (Affymetrix MoGene 1.0ST array) and data processing were done at the ImmGen Project processing center. Details of the data analysis and quality control can be found at (www.immgen.org).

Cytokine measurements

Flash frozen lung lobes from infected mice were thawed and lysed using the Bio-Rad Bio-Plex Cell Lysis Kit (Bio-Rad Laboratories, Inc., CA, USA) and a FastPrep-24 homogenizer (MP Biomedicals, CA, USA). Protein concentrations were quantified using the Pierce BCA protein assay kit (Life Technologies, CA, USA) and diluted to a concentration of 1.5 mg/mL in PBS containing BSA. Samples were analyzed using the Bio-Rad Bio-Plex Pro Mouse Ctyokine Immunoassay and a Bio-Rad Bio-Plex 200 suspension array reader.

Statistical analysis

All data are represented as mean with SEM. Comparisons of two groups within 1:1 mixed bone marrow chimeras were done with a paired student's t-test. All other comparisons were done with an unpaired student's t-test and are indicated in the figure legends. Comparisons of more than two groups were done using Holm-Šídák multiple comparisons testing following two-way ANOVA. Significance was represented by the following symbols: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and ‡P < 0.0001.

References

1. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med. 1989 Sep 1;170(3):827–45.

2. Stern AS, Podlaski FJ, Hulmes JD, Pan YC, Quinn PM, Wolitzky AG, et al. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc Natl Acad Sci USA. 1990 Sep;87(17):6808–12.

3. Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med. 1993 Apr 1;177(4):1199–204.

4. Gazzinelli RT, Hieny S, Wynn TA, Wolf S, Sher A. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. Proc Natl Acad Sci USA. 1993 Jul 1;90(13):6115–9.

5. Seder RA, Gazzinelli R, Sher A, Paul WE. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. Proc Natl Acad Sci USA. 1993 Nov 1;90(21):10188–92.

6. D'Andrea A, Rengaraju M, Valiante NM, Chehimi J, Kubin M, Aste M, et al. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J Exp Med. 1992 Nov 1;176(5):1387–98.

7. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science. 1993 Apr 23;260(5107):547–9.

8. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol. 1995 May 15;154(10):5071–9.

9. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity. 2000 Nov;13(5):715–25.

10. Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells. Immunity. 2002 Jun;16(6):779–90.

11. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature. 2007 Nov 22;450(7169):566–9.

12. Jones LL, Vignali DAA. Molecular interactions within the IL-6/IL-12 cytokine/receptor superfamily. Immunol Res. 2011 Oct;51(1):5–14.

13. Vignali DAA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. Nat Immunol. 2012 Aug;13(8):722–8.

14. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nature Reviews Immunology. 2003 Feb;3(2):133–46.

15. Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nature Reviews Immunology. 2005 Jul;5(7):521–31.

16. Hunter CA, Kastelein R. Interleukin-27: balancing protective and pathological immunity. Immunity. 2012 Dec 14;37(6):960–9.

17. Bustamante J, Boisson-Dupuis S, Abel L, Casanova J-L. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-γ immunity. Seminars in Immunology. 2014 Dec;26(6):454–70.

18. de Jong R, Altare F, Haagen IA, Elferink DG, Boer T, van Breda Vriesman PJ, et al. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. Science. 1998 May 29;280(5368):1435–8.

19. Altare F, Durandy A, Lammas D, Emile JF, Lamhamedi S, Le Deist F, et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science. 1998 May 29;280(5368):1432–5.

20. Fieschi C, Dupuis S, Catherinot E, Feinberg J, Bustamante J, Breiman A, et al. Low penetrance, broad resistance, and favorable outcome of interleukin 12 receptor beta1 deficiency: medical and immunological implications. J Exp Med. 2003 Feb 17;197(4):527–35.

21. Boisson-Dupuis S, El-Baghdadi J, Parvaneh N, Bousfiha A, Bustamante J, Feinberg J, et al. IL-12Rβ1 deficiency in two of fifty children with severe tuberculosis from Iran, Morocco, and Turkey. PLoS ONE. 2011;6(4):e18524.

22. Tabarsi P, Marjani M, Mansouri N, Farnia P, Boisson-Dupuis S, Bustamante J, et al. Lethal tuberculosis in a previously healthy adult with IL-12 receptor deficiency. J Clin Immunol. 2011 Aug;31(4):537–9.

23. Caragol I, Raspall M, Fieschi C, Feinberg J, Larrosa MN, Hernández M, et al. Clinical tuberculosis in 2 of 3 siblings with interleukin-12 receptor beta1 deficiency. Clin Infect Dis. 2003 Jul 15;37(2):302–6.

24. Ozbek N, Fieschi C, Yilmaz BT, de Beaucoudrey L, Demirhan B, Feinberg J, et al. Interleukin-12 receptor beta 1 chain deficiency in a child with disseminated tuberculosis. Clin Infect Dis. 2005 Mar 15;40(6):e55–8.

25. Reiling N, Hölscher C, Fehrenbach A, Kröger S, Kirschning CJ, Goyert S, et al. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. J Immunol. 2002 Oct 1;169(7):3480–4.

26. Bafica A, Scanga CA, Feng CG, Leifer C, Cheever A, Sher A. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. J Exp Med. 2005 Dec 19;202(12):1715–24.

27. Pompei L, Jang S, Zamlynny B, Ravikumar S, McBride A, Hickman SP, et al. Disparity in IL-12 release in dendritic cells and macrophages in response to Mycobacterium tuberculosis is due to use of distinct TLRs. J Immunol. 2007 Apr 15;178(8):5192–9.

28. Scanga CA, Bafica A, Feng CG, Cheever AW, Hieny S, Sher A. MyD88-deficient mice display a profound loss in resistance to Mycobacterium tuberculosis associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. Infection and Immunity. 2004 Apr;72(4):2400–4.

29. Fremond CM, Yeremeev V, Nicolle DM, Jacobs M, Quesniaux VF, Ryffel B. Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88. J Clin Invest. 2004 Dec;114(12):1790–9.

30. Kamath AB, Alt J, Debbabi H, Behar SM. Toll-like receptor 4-defective C3H/HeJ mice are not more susceptible than other C3H substrains to infection with Mycobacterium tuberculosis. Infection and Immunity. 2003 Jul;71(7):4112–8.

31. Sugawara I, Yamada H, Li C, Mizuno S, Takeuchi O, Akira S. Mycobacterial infection in TLR2 and TLR6 knockout mice. Microbiol Immunol. 2003;47(5):327–36.

32. Kinjo Y, Kawakami K, Uezu K, Yara S, Miyagi K, Koguchi Y, et al. Contribution of IL-18 to Th1 response and host defense against infection by Mycobacterium tuberculosis: a comparative study with IL-12p40. J Immunol. 2002 Jul 1;169(1):323–9.

33. Fremond CM, Togbe D, Doz E, Rose S, Vasseur V, Maillet I, et al. IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to Mycobacterium tuberculosis infection. J Immunol. 2007 Jul 15;179(2):1178–89.

34. Cooper AM, Roberts AD, Rhoades ER, Callahan JE, Getzy DM, Orme IM. The role of interleukin-12 in acquired immunity to Mycobacterium tuberculosis infection. Immunology. 1995 Mar;84(3):423–32.

35. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med. 1993 Dec 1;178(6):2249–54.

36. Flynn JL, Goldstein MM, Triebold KJ, Sypek J, Wolf S, Bloom BR. IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection. J Immunol. 1995 Sep 1;155(5):2515–24.

37. Cooper AM, Magram J, Ferrante J, Orme IM. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. J Exp Med. 1997 Jul 7;186(1):39–45.

38. Cooper AM, Kipnis A, Turner J, Magram J, Ferrante J, Orme IM. Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present. J Immunol. 2002 Feb 1;168(3):1322–7.

39. Mattner F, Fischer S, Guckes S, Jin S, Kaulen H, Schmitt E, et al. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. Eur J Immunol. 1993 Sep;23(9):2202–8.

40. Gillessen S, Carvajal D, Ling P, Podlaski FJ, Stremlo DL, Familletti PC, et al. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur J Immunol. 1995 Jan;25(1):200–6.

41. Gately MK, Carvajal DM, Connaughton SE, Gillessen S, Warrier RR, Kolinsky KD, et al. Interleukin-12 antagonist activity of mouse interleukin-12 p40 homodimer in vitro and in vivo. Annals of the New York Academy of Sciences. 1996 Oct 31;795:1–12.

42. Cooper AM, Khader SA. IL-12p40: an inherently agonistic cytokine. Trends in Immunology. 2007 Jan;28(1):33–8.

43. Khader SA, Pearl JE, Sakamoto K, Gilmartin L, Bell GK, Jelley-Gibbs DM, et al. IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-gamma responses if IL-12p70 is available. J Immunol. 2005 Jul 15;175(2):788–95.

44. Teixeira-Coelho M, Cruz A, Carmona J, Sousa C, Ramos-Pereira D, Saraiva AL, et al. TLR2 deficiency by compromising p19 (IL-23) expression limits Th 17 cell responses to Mycobacterium tuberculosis. Int Immunol. 2011 Feb;23(2):89–96.

45. Holscher C, Atkinson RA, Arendse B, Brown N, Myburgh E, Alber G, et al. A protective and agonistic function of IL-12p40 in mycobacterial infection. J Immunol. 2001 Dec 15;167(12):6957–66.

46. Wozniak TM, Ryan AA, Britton WJ. Interleukin-23 restores immunity to

Mycobacterium tuberculosis infection in IL-12p40-deficient mice and is not required for the development of IL-17-secreting T cell responses. J Immunol. 2006 Dec 15;177(12):8684–92.

47. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol. 2007 Apr;8(4):369–77.

48. Cruz A, Fraga AG, Fountain JJ, Rangel Moreno J, Torrado E, Saraiva M, et al. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. Journal of Experimental Medicine. 2010 Aug 2;207(8):1609–16.

49. Wozniak TM, Saunders BM, Ryan AA, Britton WJ. Mycobacterium bovis BCG-specific Th17 cells confer partial protection against Mycobacterium tuberculosis infection in the absence of gamma interferon. Infection and Immunity. 2010 Oct;78(10):4187–94.

50. Okamoto Yoshida Y, Umemura M, Yahagi A, O'Brien RL, Ikuta K, Kishihara K, et al. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. The Journal of Immunology. 2010 Apr 15;184(8):4414–22.

51. Behar SM, Carpenter SM, Booty MG, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. Seminars in Immunology. 2014 Dec;26(6):559–77.

52. Khader SA, Guglani L, Rangel Moreno J, Gopal R, Junecko BAF, Fountain JJ, et al. IL-23 is required for long-term control of Mycobacterium tuberculosis and B cell follicle formation in the infected lung. The Journal of Immunology. 2011 Nov 15;187(10):5402–7.

53. Hall AO, Silver JS, Hunter CA. The immunobiology of IL-27. Adv Immunol. 2012;115:1–44.

54. Villarino A, Hibbert L, Lieberman L, Wilson E, Mak T, Yoshida H, et al. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. Immunity. 2003 Nov;19(5):645–55.

55. Pearl JE, Khader SA, Solache A, Gilmartin L, Ghilardi N, deSauvage F, et al. IL-27 signaling compromises control of bacterial growth in mycobacteria-infected mice. J Immunol. 2004 Dec 15;173(12):7490–6.

56. Hölscher C, Hölscher A, Rückerl D, Yoshimoto T, Yoshida H, Mak T, et al. The IL-27 receptor chain WSX-1 differentially regulates antibacterial immunity and survival during experimental tuberculosis. J Immunol. 2005 Mar 15;174(6):3534–44.

57. Morishima N, Owaki T, Asakawa M, Kamiya S, Mizuguchi J, Yoshimoto T. Augmentation of effector CD8+ T cell generation with enhanced granzyme B expression by IL-27. J Immunol. 2005 Aug 1;175(3):1686–93.

58. Mayer KD, Mohrs K, Reiley W, Wittmer S, Kohlmeier JE, Pearl JE, et al. Cutting edge: T-bet and IL-27R are critical for in vivo IFN-gamma production by CD8 T cells during infection. J Immunol. 2008 Jan 15;180(2):693–7.

59. Morishima N, Mizoguchi I, Okumura M, Chiba Y, Xu M, Shimizu M, et al. A pivotal role for interleukin-27 in CD8+ T cell functions and generation of cytotoxic T lymphocytes. J Biomed Biotech. 2010;2010:605483.

60. Schneider R, Yaneva T, Beauseigle D, El-Khoury L, Arbour N. IL-27 increases the proliferation and effector functions of human naïve CD8+ T lymphocytes and promotes their development into Tc1 cells. Eur J Immunol. 2011 Jan;41(1):47–59.

61. ISAACS A, LINDENMANN J. Virus interference. I. The interferon. Proc R Soc Lond, B, Biol Sci. 1957 Sep 12;147(927):258–67.

62. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. Immunol Rev. 2004 Dec;202:8–32.

63. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. Nature Reviews Immunology. 2015 Jan 23;15(2):87–103.

64. Moreira LO, Zamboni DS. NOD1 and NOD2 Signaling in Infection and Inflammation. Front Immunol. 2012;3:328.

65. Goubau D, Deddouche S, Reis e Sousa C. Cytosolic sensing of viruses. Immunity. 2013 May 23;38(5):855–69.

66. Paludan SR, Bowie AG. Immune sensing of DNA. Immunity. 2013 May 23;38(5):870–80.

67. Moynagh PN. TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. Trends in Immunology. 2005 Sep;26(9):469–76.

68. Trinchieri G. Type I interferon: friend or foe? Journal of Experimental Medicine. 2010 Sep 27;207(10):2053–63.

69. Pandey AK, Yang Y, Jiang Z, Fortune SM, Coulombe F, Behr MA, et al. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to Mycobacterium tuberculosis. PLoS Pathog. 2009 Jul;5(7):e1000500.

70. Stanley SA, Johndrow JE, Manzanillo P, Cox JS. The Type I IFN response to infection with Mycobacterium tuberculosis requires ESX-1-mediated secretion and contributes to pathogenesis. J Immunol. 2007 Feb 14;178(5):3143–52.

71. McNab FW, Ewbank J, Rajsbaum R, Stavropoulos E, Martirosyan A, Redford PS, et al. TPL-2-ERK1/2 signaling promotes host resistance against intracellular bacterial infection by negative regulation of type I IFN production. The Journal of Immunology. 2013 Aug 15;191(4):1732–43.

72. Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ, et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res. 2005 Nov;25(11):694–701.

73. Ordway D, Henao-Tamayo M, Harton M, Palanisamy G, Troudt J, Shanley C, et al. The hypervirulent Mycobacterium tuberculosis strain HN878 induces a potent TH1 response followed by rapid down-regulation. J Immunol. 2007 Jul 1;179(1):522–31.

74. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta. Proc Natl Acad Sci USA. 2001 May 8;98(10):5752–7.

75. Antonelli LRV, Gigliotti Rothfuchs A, Gonçalves R, Roffê E, Cheever AW, Bafica A, et al. Intranasal Poly-IC treatment exacerbates tuberculosis in mice through the pulmonary recruitment of a pathogen-permissive monocyte/macrophage population. J Clin Invest. 2010 May;120(5):1674–82.

76. Redford PS, Mayer-Barber KD, McNab FW, Stavropoulos E, Wack A, Sher A, et al. Influenza A virus impairs control of Mycobacterium tuberculosis coinfection through a type I interferon receptor-dependent pathway. J INFECT DIS. 2014 Jan 15;209(2):270–4.

77. Berry MPR, Graham CM, McNab FW, Xu Z, Bloch SAA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010 Aug 19;466(7309):973–7.

78. Ottenhoff THM, Dass RH, Yang N, Zhang MM, Wong HEE, Sahiratmadja E, et al. Genome-wide expression profiling identifies type 1 interferon response pathways in active tuberculosis. PLoS ONE. 2012;7(9):e45839.

79. Mariotti S, Teloni R, Iona E, Fattorini L, Romagnoli G, Gagliardi MC, et al. Mycobacterium tuberculosis diverts alpha interferon-induced monocyte differentiation from dendritic cells into immunoprivileged macrophage-like host cells. Infection and Immunity. 2004 Aug;72(8):4385–92.

80. de Paus RA, van Wengen A, Schmidt I, Visser M, Verdegaal EME, van Dissel JT, et al. Inhibition of the type I immune responses of human monocytes by IFN-α and IFN-β. Cytokine. 2013 Feb;61(2):645–55.

81. McNab FW, Ewbank J, Howes A, Moreira-Teixeira L, Martirosyan A, Ghilardi N,

et al. Type I IFN induces IL-10 production in an IL-27-independent manner and blocks responsiveness to IFN-γ for production of IL-12 and bacterial killing in Mycobacterium tuberculosis-infected macrophages. The Journal of Immunology. 2014 Oct 1;193(7):3600–12.

82. Teles RMB, Graeber TG, Krutzik SR, Montoya D, Schenk M, Lee DJ, et al. Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. Science. 2013 Mar 22;339(6126):1448–53.

83. Bhatt K, Uzelac A, Mathur S, McBride A, Potian J, Salgame P. B7 costimulation is critical for host control of chronic Mycobacterium tuberculosis infection. The Journal of Immunology. 2009 Mar 15;182(6):3793–800.

84. Bhatt K, Kim A, Kim A, Mathur S, Salgame P. Equivalent functions for B7.1 and B7.2 costimulation in mediating host resistance to Mycobacterium tuberculosis. CELLULAR IMMUNOLOGY. 2013 Sep;285(1-2):69–75.

85. Lancioni CL, Li Q, Thomas JJ, Ding X, Thiel B, Drage MG, et al. Mycobacterium tuberculosis lipoproteins directly regulate human memory CD4(+) T cell activation via Toll-like receptors 1 and 2. Infection and Immunity. 2011 Feb;79(2):663–73.

86. Reba SM, Li Q, Onwuzulike S, Ding X, Karim AF, Hernandez Y, et al. TLR2 engagement on CD4(+) T cells enhances effector functions and protective responses to Mycobacterium tuberculosis. Eur J Immunol. 2014 May;44(5):1410–21.

87. Li Q, Ding X, Thomas JJ, Harding CV, Pecora ND, Ziady AG, et al. Rv2468c, a novel Mycobacterium tuberculosis protein that costimulates human CD4+ T cells through VLA-5. Journal of Leukocyte Biology. 2012 Feb;91(2):311–20.

88. Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK, et al. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J Immunol. 1999 Mar 15;162(6):3256–62.

89. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol. 2005 Apr 15;174(8):4465–9.

90. Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, et al. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev. 2006 May 31;211:81–92.

91. Schmidt CS, Mescher MF. Adjuvant effect of IL-12: conversion of peptide antigen administration from tolerizing to immunizing for CD8+ T cells in vivo. J Immunol. 1999 Sep 1;163(5):2561–7.

92. Mainetti LE, Rozados VR, Rossa A, Bonfil RD, Scharovsky OG. Antitumoral and

antimetastatic effects of metronomic chemotherapy with cyclophosphamide combined with celecoxib on murine mammary adenocarcinomas. J Cancer Res Clin Oncol. 2010 Mar 27;137(1):151–63.

93. Sikora AG, Jaffarzad N, Hailemichael Y, Gelbard A, Stonier SW, Schluns KS, et al. IFN-alpha enhances peptide vaccine-induced CD8+ T cell numbers, effector function, and antitumor activity. The Journal of Immunology. 2009 Jun 15;182(12):7398–407.

94. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med. 2005 Sep 5;202(5):637–50.

95. Aichele P, Unsoeld H, Koschella M, Schweier O, Kalinke U, Vucikuja S. CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. J Immunol. 2006 Apr 15;176(8):4525–9.

96. Keppler SJ, Theil K, Vucikuja S, Aichele P. Effector T-cell differentiation during viral and bacterial infections: Role of direct IL-12 signals for cell fate decision of CD8+ T cells. Eur J Immunol. 2009 Jun 22;39(7):1774–83.

97. Starbeck-Miller GR, Xue H-H, Harty JT. IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo. Journal of Experimental Medicine. 2014 Jan 13;211(1):105–20.

98. Keppler SJ, Rosenits K, Koegl T, Vucikuja S, Aichele P. Signal 3 cytokines as modulators of primary immune responses during infections: the interplay of type I IFN and IL-12 in CD8 T cell responses. PLoS ONE. 2012;7(7):e40865.

99. Wilson DC, Matthews S, Yap GS. IL-12 signaling drives CD8+ T cell IFN-gamma production and differentiation of KLRG1+ effector subpopulations during Toxoplasma gondii Infection. J Immunol. 2008 May 1;180(9):5935–45.

100. Xiao Z, Casey KA, Jameson SC, Curtsinger JM, Mescher MF. Programming for CD8 T cell memory development requires IL-12 or type I IFN. The Journal of Immunology. 2009 Mar 1;182(5):2786–94.

101. Curtsinger JM, Mescher MF. Inflammatory cytokines as a third signal for T cell activation. Current Opinion in Immunology. 2010 Jun;22(3):333–40.

102. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol. 2003 Dec;4(12):1191–8.

103. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, et al. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity. 2007 Aug;27(2):281–95.

104. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. Journal of Experimental Medicine. 2008 Mar 17;205(3):625–40.

105. Lefrançois L, Obar JJ. Once a killer, always a killer: from cytotoxic T cell to memory cell. Immunol Rev. 2010 May;235(1):206–18.

106. Obar JJ, Molloy MJ, Jellison ER, Stoklasek TA, Zhang W, Usherwood EJ, et al. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. Proceedings of the National Academy of Sciences. 2010 Jan 5;107(1):193–8.

107. Cui W, Kaech SM. Generation of effector CD8+ T cells and their conversion to memory T cells. Immunol Rev. 2010 Jul;236:151–66.

108. Obar JJ, Jellison ER, Sheridan BS, Blair DA, Pham Q-M, Zickovich JM, et al. Pathogen-induced inflammatory environment controls effector and memory CD8+ T cell differentiation. The Journal of Immunology. 2011 Nov 15;187(10):4967–78.

109. Olson JA, McDonald-Hyman C, Jameson SC, Hamilton SE. Effector-like CD8(+) T Cells in the Memory Population Mediate Potent Protective Immunity. Immunity. 2013 Jun 27;38(6):1250–60.

110. Plumlee CR, Sheridan BS, Cicek BB, Lefrançois L. Environmental cues dictate the fate of individual CD8(+) T cells responding to infection. Immunity. 2013 Aug 22;39(2):347–56.

111. Badovinac VP, Harty JT. Manipulating the rate of memory CD8+ T cell generation after acute infection. J Immunol. 2007 Jul 1;179(1):53–63.

112. Hand TW, Morre M, Kaech SM. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. Proc Natl Acad Sci USA. 2007 Jul 10;104(28):11730–5.

113. Keppler SJ, Aichele P. Signal 3 requirement for memory CD8+ T-cell activation is determined by the infectious pathogen. Eur J Immunol. 2011 Sep 26;41(11):3176–86.

114. Pennock ND, Gapin L, Kedl RM. IL-27 is required for shaping the magnitude, affinity distribution, and memory of T cells responding to subunit immunization. Proceedings of the National Academy of Sciences. 2014 Nov 18;111(46):16472–7.

115. Heng TSP, Painter MW, Immunological Genome Project Consortium. The Immunological Genome Project: networks of gene expression in immune cells. Nat Immunol. 2008 Oct;9(10):1091–4.

116. Mayer-Barber KD, Andrade BB, Oland SD, Amaral EP, Barber DL, Gonzales J, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. Nature. 2014 Jul 3;511(7507):99–103.

117. Woodworth JS, Wu Y, Behar SM. Mycobacterium tuberculosis-specific CD8+ T cells require perforin to kill target cells and provide protection in vivo. The Journal of Immunology. 2008 Dec 15;181(12):8595–603.

118. Woodworth JSM, Behar SM. Mycobacterium tuberculosis-specific CD8+ T cells and their role in immunity. Crit Rev Immunol. 2006;26(4):317–52.

119. Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. Infection and Immunity. 2002 Aug;70(8):4501–9.

120. Sher A, Collazzo C, Scanga C, Jankovic D, Yap G, Aliberti J. Induction and regulation of IL-12-dependent host resistance to Toxoplasma gondii. Immunol Res. 2003;27(2-3):521–8.

121. Brennan AJ, House IG, Oliaro J, Ramsbottom KM, Hagn M, Yagita H, et al. A method for detecting intracellular perforin in mouse lymphocytes. The Journal of Immunology. 2014 Dec 1;193(11):5744–50.

122. Casey KA, Mescher MF. IL-21 promotes differentiation of naive CD8 T cells to a unique effector phenotype. J Immunol. 2007 Jun 15;178(12):7640–8.

123. Müller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, et al. Functional role of type I and type II interferons in antiviral defense. Science. 1994 Jun 24;264(5167):1918–21.

124. Marshall HD, Urban SL, Welsh RM. Virus-induced transient immune suppression and the inhibition of T cell proliferation by type I interferon. Journal of Virology. 2011 Jun;85(12):5929–39.

125. Holst J, Szymczak-Workman AL, Vignali KM, Burton AR, Workman CJ, Vignali DAA. Generation of T-cell receptor retrogenic mice. Nat Protoc. 2006;1(1):406–17.

Chapter 3:

$CD8^+$ T cells require direct IL-21 signaling during tuberculosis

This work is adapted from an untitled and unpublished manuscript. The work presented included assistance from the following individuals: Cláudio Nunes-Alves, and Pushpa Jayaraman (University of Massachusetts Medical School, Worcester, MA).

Abstract

IL-21 is primarily produced by activated $CD4^+$ T cells and is a mediator of CD4 help to both $CD8^+$ T cells and B cells. During chronic viral infections, IL-21 acts directly on $CD8^+$ T cells to support expansion and cytokine production in addition to actively inhibiting $CD8^+$ T cell exhaustion. Though IL-21 is essential during many chronic infections, it has never been closely examined during tuberculosis. Here, we define an essential role for IL-21 during the immune response to tuberculosis and establish it as a mediator of host resistance.

We address the impact of IL-21 on the immune response with the use of mixed bone marrow chimeras. These experiments indicate IL-21R is required on $CD8^+$ T cells for efficient expansion and acquisition of effector functions during tuberculosis. Using the adoptive transfer of Ag-specific IL-$21R^{-/-}$ $CD8^+$ T cells, we demonstrate that $CD8^+$ T cells require IL-21 for expansion both in the lymph node during priming and in the lungs. This suggests IL-21 acts as “signal 3” cytokines and is required at all stages of the $CD8^+$ T cell response. These observations highlight the vital role of $CD4^+$ T cell help during tuberculosis, as $CD4^+$ T cells are the primary source of IL-21.

We also show preliminary data examining the susceptibility of IL-$21R^{-/-}$ mice. Four weeks after aerosol infection, IL-$21R^{-/-}$ mice have reduced numbers of $CD8^+$ T cells, but otherwise have an intact immune response. At later time points, $CD8^+$ T cells show signs of exhaustion, including increased PD-1 expression and decreased TNF production. By sixteen weeks post-infection, IL-$21R^{-/-}$ mice failed to control bacterial growth and have reduced numbers of both $CD4^+$ and $CD8^+$ T cells in the lungs. These

data indicate IL-21R signaling supports resistance during chronic infection and is essential to maintain both $CD4^+$ and $CD8^+$ T cell responses. We are currently investigating the mechanism behind the decline in $CD4^+$ T cell numbers observed in IL-$21R^{-/-}$ mice. During tuberculosis, IL-21 is essential for $CD8^+$ T cell responses and it influences host resistance through multiple mechanisms.

Introduction

Basic biology of IL-21

The common receptor gamma-chain (γc) family of cytokines consist of IL-2, IL-7, IL-9, IL-15, and IL-21, and all members share the common gamma-chain (γc) as a component of their individual receptors (1). The γc was first cloned in 1992 as a component of the IL-2 receptor, thus IL-2 is the prototypical member of this cytokine family (2). Cytokines that signal through γc support the survival and function of a variety of immune cells, a fact that is highlighted by the human disease X-linked severe combined immunodeficiency (XSCID). Patients with XSCID lack T cells and NK cells and have nonfunctioning B cells as a result of mutations in the gene encoding γc (*IL2RG*) (3,4). In particular, many cytokines that utilize γc are important for $CD8^+$ T cells. IL-2 can support $CD8^+$ T cell expansion both during priming and memory recall (5-11). IL-7 is required for the homeostatic proliferation of naïve $CD8^+$ T cells and supports the survival of memory cells (12,13). IL-15 is necessary for SLEC survival in addition to the proliferative maintenance of memory $CD8^+$ T cells (14-19). Thus, IL-2 and IL-15 are essential for effector $CD8^+$ T responses and IL-7 and IL-15 maintain memory populations.

The IL-21 receptor (IL-21R) was first discovered in 2000, and the identification of its ligand, IL-21, soon followed (20,21). In 2001, γc was recognized as the other essential component for IL-21R signal transduction, placing IL-21 in the γc family of cytokines (22,23). IL-21R is predominantly expressed on B, $CD4^+$ T, $CD8^+$ T, NKT, and NK cells, though B cells express the highest amounts by far (24). Early studies suggest

IL-21R is expressed at relatively low levels on resting T cells and is upregulated upon TCR stimulation or IL-21 signaling (24-27). IL-21R expression has also been noted on DCs, macrophages, and some non-hematopoietic cells (28-30). IL-21 is mainly produced by $CD4^+$ T and NKT cells, but studies have also noted production by $CD8^+$ T and γδ T cells (21,24,31-36). Interestingly, NKT cells, but not $CD4^+$ T cells, produce high amounts of IL-21 in response to activation with bacillus Calmette-Guérin (BCG) (32). Since it's discovery, IL-21 has been ascribed important roles during infection, autoimmunity, and cancer (4,37). Largely, its effects are pro-inflammatory and primarily influence B, $CD4^+$ T, and $CD8^+$ T cell functions.

IL-21 supports plasma cell generation and antibody production

In addition to lacking T and NK cells, XSCID patients have nonfunctional B cells. One mechanism behind this dysfunction was revealed with the discovery that IL-$21R^{-/-}$ mice have dramatically impaired Ag-specific IgG1 production in response to immunization and infection (38). IL-21 is not necessary for B cell development, but multiple studies have implicated a role for it in immunoglobulin production and plasma cell generation (38-42). It is also important for the formation of germinal centers (GCs). GCs are sites of B cell expansion, somatic hypermutation, and affinity maturation. They are also necessary for the formation of memory B cells and plasma cells. IL-21 supports GC formation by acting directly on B cells to promote the expression of the transcription factor Bcl-6; however, GC formation can occur independently of IL-21 in some contexts (43-45). The source of IL-21 during GC formation is T follicular helper cells (Tfh), a

subset of $CD4^+$ T cells that assist in forming and maintain GCs. These cells, along with T_H17 cells, are major producers of IL-21.

IL-21 and $CD4^+$ T cell subsets

Broadly, IL-21 influences $CD4^+$ T cells by promoting T_H2 polarization while also limiting T_H1 polarization, but it is also affects other T helper subsets. (46-49). Tfh cells are important for B cell help and are among the greatest producers of IL-21 (37,50-52). Tfh cells also respond directly to IL-21, though its role in their development is somewhat controversial. IL-21 is not absolutely required for Tfh generation, but it synergizes with other cytokines, mainly IL-6, to promote Tfh differentiation and function (52-55). IL-21 also affects both regulatory T cells (Tregs) and T_H17 cells. It antagonizes the expansion of regulatory T cells (Tregs) through direct signaling and indirectly by suppressing IL-2 levels (56,57). This ability to suppress Treg expansion inherently supports the generation of T_H17 cells. Similar to Tfh cells, T_H17 cells make and respond to IL-21. It is not required for T_H17 development *in vivo*, but promotes the expansion of existing T_H17 cells (33,37,58-62). IL-21 assists in maintaining T_H17 cells by inducing IL-23R expression, and IL-23 signaling subsequently preserves the T_H17 phenotype (62). $CD4^+$ T cells are the major sources of IL-21 during a variety of diseases states, thus they mediate much of its effects *in vivo*.

IL-21 and $CD8^+$ T cells

Similar to B cells, $CD8^+$ T cells respond to IL-21 and are major targets of its effects (24). $CD8^+$ T cells can produce IL-21 in some settings, and it may act in an

autocrine manner to promote effector function (63). However, the relevance of $CD8^+$ T cell-derived IL-21 is poorly understood, and most studies implicate $CD4^+$ T cells as the primary source of this cytokine. IL-21 is not essential for naïve $CD8^+$ T cell development, but it can profoundly shape primary and, to some extent, memory responses. Indirectly, IL-21 supports $CD8^+$ T cell proliferation and effector function by restricting the expansion of Tregs (57,64); however, its effects are largely mediated by direct signaling on $CD8^+$ T cells. Numerous *in vitro* studies with both murine and human cells have demonstrated a direct stimulatory effect on $CD8^+$ T cell expansion (65-68). IL-21 can synergize with IL-15, and to a lesser extent IL-7, to promote proliferation, and it independently supports cell survival (67,69-71). In experiments with artificial antigen presenting cells (aAPCs), IL-21 facilitated naïve $CD8^+$ T cell priming and cytotoxic T lymphocyte (CTL) differentiation when combined with IL-2, TCR ligation, and co-stimulation (72). Thus, IL-21 may act as a signal 3 cytokine; however, this has never been fully addressed *in vivo*. $CD4^+$ T cell-derived IL-21 can also rescue the expansion of naïve $CD8^+$ T cells under priming conditions where IL-2 is depleted, suggesting IL-21 can stand-in for IL-2 in some contexts (73). In addition to its effects on primary expansion, IL-21 can support memory responses, though there are conflicting reports regarding its role in memory development. Following the clearance of certain acute strains of lymphocytic choriomeningitis virus (LCMV), memory IL-$21R^{-/-}$ $CD8^+$ T cells expand poorly upon rechallenge (74). During vaccinia virus (VV) infection, IL-$21R^{-/-}$ $CD8^+$ T cells are unable to survive the contraction phase and fail to form a memory pool (71). Still, other reports show IL-21 is dispensable for memory responses, and this role likely varies between pathogens (34).

IL-21 augments effector function by promoting IFN-γ, granzyme B, and perforin production, and it also enhances cytotoxicity (65-67,75,76). In mouse models of tumor growth, IL-21 therapy potently limits tumor size in a manner dependent on $CD8^+$ T cell-mediated killing (66,67,77). T-bet is essential for the IL-21-driven differentiation of CTLs, and IL-21 directly induces T-bet expression (76). Overall, IL-21 aids in the expansion of $CD8^+$ T cells and promotes their function. Given that $CD4^+$ T cells are the major source of IL-21, this underscores the importance of $CD4^+$ T cell help in generating potent effector $CD8^+$ T cells.

The role of IL-21 during infection

A dramatic role for IL-21 during infection was revealed when comparing acute and chronic viral clones. In the absence of IL-21, mice are capable of clearing acute strains of LCMV; however, they are unable to control chronic LCMV infection and develop high viral titers. (34,78,79). Further analysis revealed that the loss of IL-21 severely diminishes the number of virus-specific $CD8^+$ T cells at later time points during chronic infection. IL-21 is dispensable for the initial response to LCMV but is required to maintain $CD8^+$ T cell numbers long term. Experiments with mixed bone marrow chimeras demonstrated that IL-21 acts directly on $CD8^+$ T cells and appears to promote continuous proliferation throughout infection (34).

In addition to maintaining $CD8^+$ T cell numbers, IL-21 is required to prevent $CD8^+$ T cell exhaustion. In the absence of IL-21, $CD8^+$ T cells produced less IFN-γ and IL-2 and express high amounts of the exhaustion marker programmed death 1, PD-1 (34,79). Thus, IL-21 is essential to maintain $CD8^+$ T cell numbers and prevent

exhaustion during chronic disease. $CD4^+$ T cells are the major source of IL-21 during LCMV infection and produce it in an antigen specific-manner. When infected with LCMV, $CD4^{-/-}$ mice are also unable to control viral replication and develop severe $CD8^+$ T cell exhaustion (80-82). Simply treating $CD4^{-/-}$ mice with exogenous IL-21 is enough to rescue $CD8^+$ T cell expansion and cytokine production, subsequently reducing viral titers (79). This experiment illustrates the importance of $CD4^+$ T cell help during infection and implicates IL-21 as an essential mediator of this help.

IL-21 has important roles in the adaptive immune response to a number of pathogens. Unlike acute LCMV, $CD4^+$ T cell-derived IL-21 is essential for $CD8^+$ T cells expansion and IFN-γ production during acute vaccinia virus infection (71). In this system, IL-21 was not needed to support the activation or proliferation of $CD8^+$ T cells but instead promoted cell survival. This ability to support survival proved essential through the contraction phase, and a memory pool failed to form in the absence of IL-21 (71). During chronic toxoplasmic encephalitis, IL-21 supports $CD8^+$ T cell cytokine production and survival in the brain (83). Surprisingly, IL-21 is dispensable for $CD8^+$ T cell responses during *Listeria monocytogenes* infection. IL-$21^{-/-}$ mice have unaltered $CD8^+$ T cell expansion and overall IFN-γ-production (84). Furthermore, IL-$21^{-/-}$ mice have comparable bacterial burdens and generate protective memory responses. *L. monocytogenes* is an acute bacterial pathogen, thus it may be more analogous to acute LCMV infection where IL-21 is also unnecessary.

Loss of IL-21 often leads to B cell dysfunction during infection, and this is frequently associated with increased susceptibility. Plasma cell generation and IgG production are reduced in IL-$21R^{-/-}$ mice infected with the gut-dwelling roundworm

Heligmosomoides polygyrus (45). The loss of IL-21 in this system ultimately compromises protective immunity, and unlike WT mice, IL-21R$^{-/-}$ mice cannot control reinfection with this pathogen. Decreased IgG titers and reduced plasma cell numbers are also observed when IL-21R$^{-/-}$ mice are infected with LCMV, influenza, and vesicular stomatitis virus (VSV) (54). In these infections, IL-21R is required on both B cells and T cells for optimal antibody production. B cell dysfunction was also noted during chronic toxoplasmic encephalitis, and mice showed reduced IgG production and GC B cells (83). IL-21$^{-/-}$ mice ultimately succumb early to *T. gondii* infection, but the precise mechanism for this remains unclear. Finally, a recent study in malaria demonstrated a key role for IL-21 in limiting parasitemia during chronic infection, and this was again associated with reduced IgG levels (85). In this system, resistance depended on IL-21 production by T cells as well as IL-21R expression on B cells. Thus, T cell-derived IL-21 acted directly on B cells to promote IgG production. IL-21 was further necessary for chloroquine-cured mice to control parasitemia upon reinfection. In summary, IL-21 is most important during chronic infections and can mediate susceptibility by influencing both CD8^{+} T cells and B cells.

Summary

IL-21 is an important cytokine during multiple chronic infections and can have broad impacts on the adaptive immune response. Currently, the role of IL-21 during chronic bacterial infections, including tuberculosis, is unknown. IL-21 mRNA is enriched in the lung lesions of patients with active tuberculosis, implicating it as a component of the local immune response to Mtb (86). Furthermore, plasma levels of IL-21 are

differentially associated with active pulmonary tuberculosis in adult and pediatric patients. In children with active pulmonary tuberculosis, plasma IL-21 is increased relative to health controls (87). Adults with active disease have decreased levels of circulating IL-21 when compared to latently infected individuals (88), suggesting high IL-21 is associated with controlled disease. There is a single published account of bacterial burden in IL-21$^{-/-}$ mice following aerosol infection with H37Rv (89). In this study, IL-21$^{-/-}$ mice controlled bacterial growth comparably to WT animals; however, there was no detailed analysis of their immune response.

Because IL-21 is essential for CD8^{+} T cell responses during chronic viral infection, we hypothesize that it is necessary to support CD8^{+} T cell expansion and function during tuberculosis. Previously, we demonstrated the importance of direct IL-12 signaling on CD8^{+} T cells during Mtb infection (Chapter 2). Despite the critical role of IL-12, CD8^{+} T cells can still be primed in its absence, suggesting there are additional signals supporting CD8^{+} T cell responses. We believe IL-21 is a strong candidate for this role.

We first address the impact of IL-21 on the immune response with the use of mixed bone marrow chimeras. These experiments indicate IL-21R is required on CD8^{+} T cells for efficient expansion and the acquisition of effector functions during tuberculosis. Using the adoptive transfer of naïve Ag-specific IL-21R$^{-/-}$ CD8^{+} T cells, we demonstrate that CD8^{+} T cells require IL-21 for expansion in both the lymph node and the lungs. This suggests IL-21 is similar to other signal 3 cytokines and is required at all stages of the CD8^{+} T cell response. As a signal 3 cytokine, IL-21 is comparable to IL-12

in its ability to promote expansion and effector function, underscoring the importance of IL-21 signaling.

Finally, we show preliminary data examining the susceptibility of IL-21R$^{-/-}$ mice. Following aerosol infection, IL-21R$^{-/-}$ mice initially have no immune defects beyond reduced CD8^{+} T cell numbers and have comparable bacterial burdens at week four post-infection. At week sixteen, we examined CD8^{+} T cells for signs of exhaustion and discovered a dramatic increase in PD-1 expression. This was accompanied by decreased TNF production, suggesting IL-21 is required to prevent CD8^{+} T cell exhaustion during tuberculosis. Additionally, IL-21R$^{-/-}$ mice failed to control bacterial growth at week 16 and had reduced numbers of both CD4^{+} and CD8^{+} T cells in the lungs. This indicates IL-21R supports resistance during chronic infection and is essential to maintain both CD4^{+} and CD8^{+} T cells. Overall, these findings detail the importance of IL-21 during chronic tuberculosis. In particular, we believe IL-21 has relevance for generating protective CD8^{+} T cells through vaccination.

Results

$CD4^+$ and $CD8^+$ T cells constitutively express the IL-21 receptor, and $CD4^+$ T cells increase IL-21 expression following Mtb infection.

Using the microarray gene expression analysis discussed in Chapter 2, we examined our dataset for the expression of IL-21 and it's receptor in lung $CD4^+$ and $CD8^+$ T cells at multiple time points following Mtb infection. Detectable expression of the IL-21 receptor, *I21r*, was observed in both $CD4^+$ and a $CD8^+$ T cells throughout the experiment and was not influenced by Mtb infection (*Figure 3-1*). Some *in vitro* studies have suggested TCR stimulation increases IL-21R expression, but steady-state expression of IL-21R has been noted on $CD4^+$ and $CD8^+$ T cells during chronic *T. gondii* infection (83). Our transcriptional analysis was performed on bulk T cells, thus we cannot comment if IL-21R expression is altered on antigen specific-cells. The expression of the cytokine, *IL21*, was minimal in naïve $CD4^+$ T cells and was upregulated following Mtb infection (*Figure 3-1*). *IL21* expression remained detectable in $CD4^+$ T cells throughout infection but was never induced to detectable levels in $CD8^+$ T cells (*Figure 3-1*). These data indicate that $CD4^+$ T cells are capable of producing IL-21 during tuberculosis and that $CD8^+$ T cells have the capacity to respond to this form of $CD4^+$ T cell help.

IL-21 augments $CD8^+$ T cell expansion during tuberculosis.

To examine the role of IL-21R on $CD8^+$ T cells, we generated 1:1 mixed bone marrow chimeras with WT ($CD45.1^+$) and IL-$21R^{-/-}$ ($CD45.2^+$) bone marrow. Following

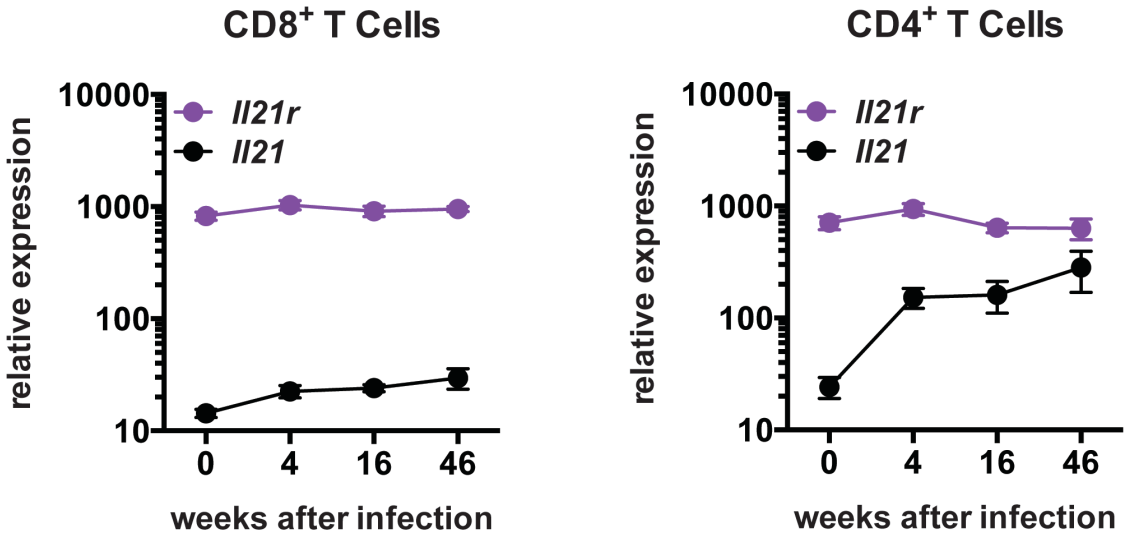

Figure 3-1. CD4$^+$ and CD8$^+$ T cells constitutively express the IL-21 receptor, and CD4$^+$ T cells increase IL-21 expression following Mtb infection.

Relative expression of the indicated transcripts in sorted CD8$^+$ (Left) and CD4$^+$ (Right) T cells purified from the lungs of uninfected mice at time 0, as well as 4, 16, and 46 weeks after low dose aerosol infection. At each time point, lung homogenates were pooled from three mice prior to magnetic bead purification and cell sorting. Replicates reflect three independent infections, each with three pooled mice. For relative expression, values >120 have a 95% probability or greater of true expression and values <47 have 95% probability or greater of being silent.

reconstitution, mice had roughly equivalent amounts of WT and IL-21R$^{-/-}$ CD4^{+} and CD8^{+} T cells, indicating that IL-21R$^{-/-}$ T cells developed normally in uninfected mice (data not shown). CD8^{+} T cells were tracked before and after infection in the same mouse by measuring the ratio of WT to IL-21R$^{-/-}$ cells in the blood. By four weeks post infection, IL-21R$^{-/-}$ CD8^{+} T cells were reduced in the blood relative to WT cells (*Figure 3-2*). During the same time frame, the ratio of WT to IL-21R$^{-/-}$ CD4^{+} T cells remained unaltered, suggesting that IL-21 signaling in dispensable for CD4^{+} T cell expansion during tuberculosis (data not show). In the lungs, IL-21R$^{-/-}$ CD8^{+} T cells accounted for less than 20% of the total CD8^{+} T cell response (*Figure 3-3A and 3-3B*). Tetramer staining for TB10.4$_{4-11}$-specific CD8^{+} T cells revealed that IL-21R$^{-/-}$ CD8^{+} T cells were equally underrepresented in this antigen-specific population (*Figure 3-3C*). These data are the first to implicate IL-21 as a major regulator of CD8^{+} T cell responses during tuberculosis, and this observation underscores the importance of CD4^{+} T cell help.

IL-21 affects CD8^{+} T cell differentiation and the acquisition of effector functions.

Using the 1:1 mixed bone marrow chimera modal, we examined the role IL-21 in CD8^{+} T cell differentiation and function during tuberculosis. KLRG1 and CD127 were used as markers to assess the differentiation of TB10.4$_{4-11}$-specific CD8^{+} T cells from the lungs. Though some differences were observed, IL-21 did not have the dramatic impact on differentiation seen with IL-12 (Chapter 2). Antigen specific IL-21R$^{-/-}$ CD8^{+} T cells showed a small but consistent increase in the percentage of KLRG1^{Hi} short-lived effector cells (SLEC) accompanied by a decrease in double negative early effector cells (EEC) (*Figure 3-4*). The functional relevance of a minor increase in the SLEC population

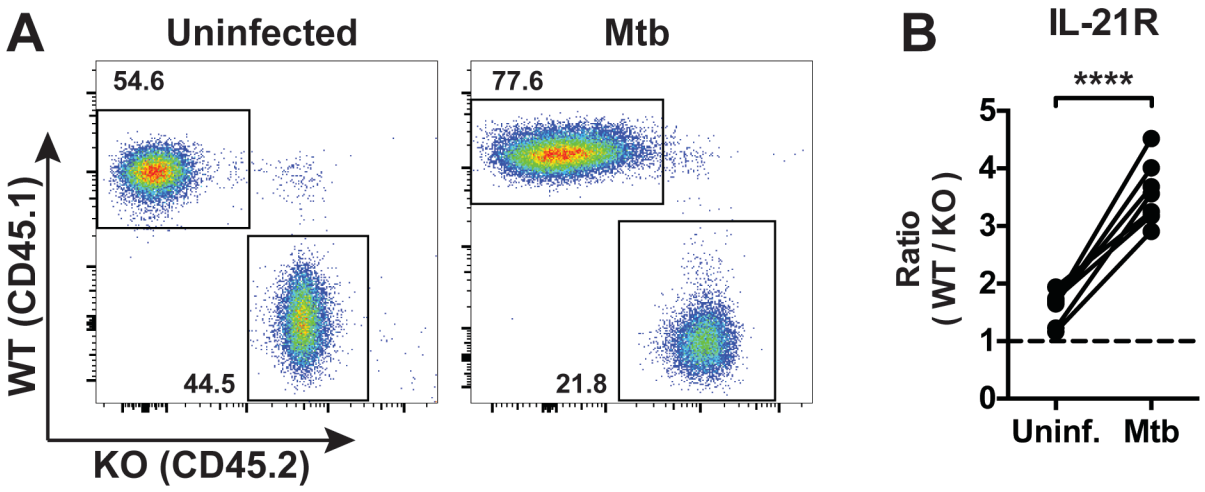

Figure 3-2. IL-21 augments CD8$^+$ T cell expansion following Mtb infection.

(A) Representative flow cytometry plots of CD3$^+$ CD8$^+$ T cells from the blood of the same 1:1 (WT:IL-21R$^{-/-}$) mixed bone marrow chimera before and four weeks after infection with Mtb. (B) The ratio of WT to KO CD8$^+$ T cells in the 1:1 chimeras before and after infection (n = 8 mice per group). ****P < 0.0001 (paired Student's t-test). Data are representative of three independent experiments.

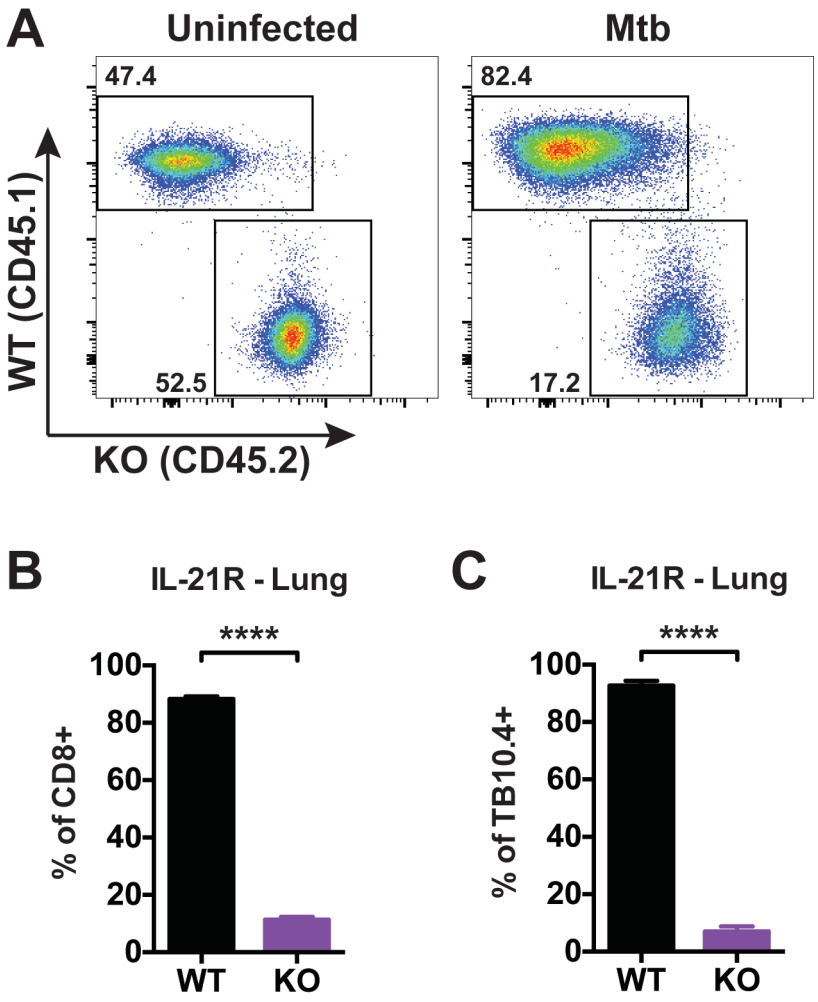

Figure 3-3. IL-21 signaling is required for the accumulation of antigen-specific CD8$^+$ T cells in the lungs during Mtb infection.

(A) Representative flow cytometry plots of CD3$^+$ CD8$^+$ T cells from the lungs of a 1:1 mixed bone marrow chimera left uninfected or one four weeks post-infection. (B) The percentage of CD3$^+$ CD8$^+$ T cells that are WT (CD45.1$^+$) or IL-21R$^{-/-}$ (CD45.2$^+$) in the lungs of infected chimeras. (C) The percentage of TB10.4-specific CD3$^+$ CD8$^+$ T cells that are WT or IL-21R$^{-/-}$ in the lungs of infected chimeras. Each bar represents the mean ± SEM (n = 8 mice per group) ****P < 0.0001 (paired Student's t-test). Data are representative of three independent experiments.

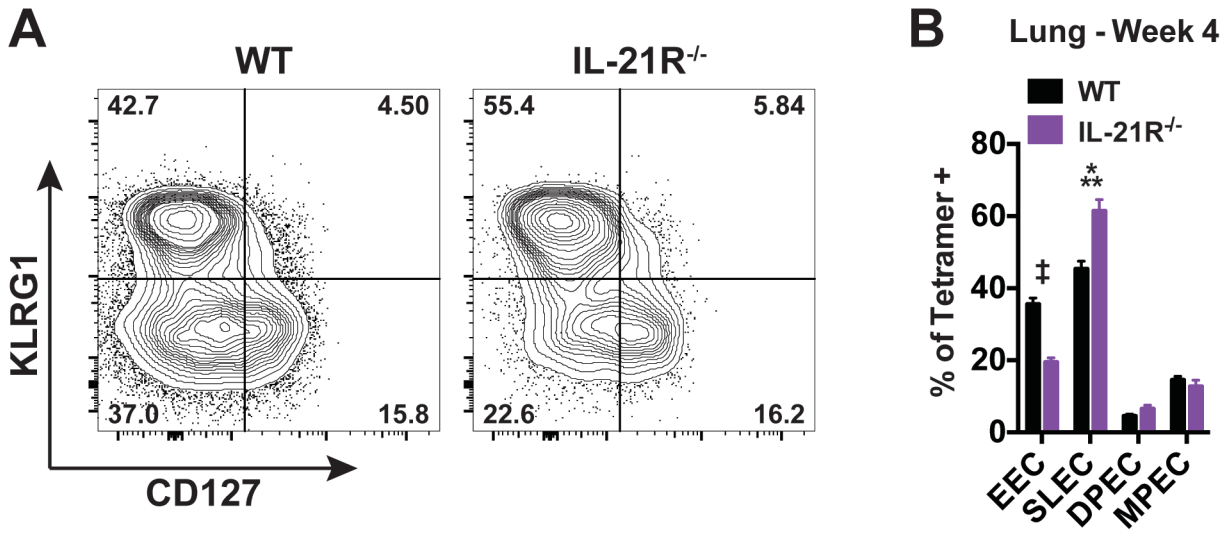

Figure 3-4. An increased percentage of IL-21R$^{-/-}$ CD8^{+} T cells become terminally differentiated short-lived effect cells (SLECs).

(A) Representative flow cytometry plots of lung CD8^{+} TB10.4^{+} T cells of the indicated genotype 4 weeks after infection. (B) Phenotypic analysis of CD8^{+} TB10.4^{+} cells based on KLRG1 and CD127 staining (EEC – Early Effector Cells; SLEC – Short-Lived Effector Cell; DPEC – Double Positive Effector Cell; MPEC – Memory Precursor Effector Cell). Each bar represents the mean ± SEM (n = 10 mice per group) ***P < 0.001, ‡P < 0.0001 (paired Student's t-test). Data are representative of two independent experiments.

is challenging to interpret; however, this may suggest IL-21 has a minor role in inhibiting the terminal differentiation of $CD8^+$ T cells. IL-12 and IL-27 were shown to drive $CD8^+$ T cell expansion and increase SLEC formation (Chapter 2). In this way, IL-21 is unique in that it dramatically influences $CD8^+$ T cell expansion without driving terminal differentiation.

Lung cells from 1:1 mixed bone marrow chimeras were stimulated *ex vivo* with the immunodominant $TB10.4_{4-11}$ peptide and intracellular cytokine staining was done to measure the percentage of $CD8^+$ T cells making IFN-γ and TNF. As controls, lung cells were left unstimulated or stimulated with anti-CD3 and anti-CD28. Significant levels of cytokine production were not observed in the unstimulated samples (data not shown). Following $TB10.4_{4-11}$ peptide stimulation, fewer IL-$21R^{-/-}$ $CD8^+$ T cells were positive for IFN-γ or double positive for IFN-γ and TNF (*Figure 3-5A and 3-5B*). In total, a smaller percentage of IL-$21R^{-/-}$ $CD8^+$ T cells were positive for either IFN-γ or TNF following TB10.4 or anti-CD3/anti-CD28 stimulation (*Figure 3-5C and 3-5D*). To address cytolytic function, we performed intracellular staining for granzyme B, and IL-$21R^{-/-}$ $CD8^+$ T cells showed reduced levels (*Figure 3-5E*). These findings support a role for IL-21 in promoting cytokine production and possibly cytolytic activity in $CD8^+$ T cells during tuberculosis.

IL-21 supports $CD8^+$ T cell expansion in the lymph node and lungs.

Given the dramatic impact of IL-21 on $CD8^+$ T cell expansion, we examined if IL-21 is directly involved in $CD8^+$ T cell priming following Mtb infection. *In vitro*, IL-21 can act as a signal 3 cytokine and is sufficient to prime naïve $CD8^+$ T cells when combined

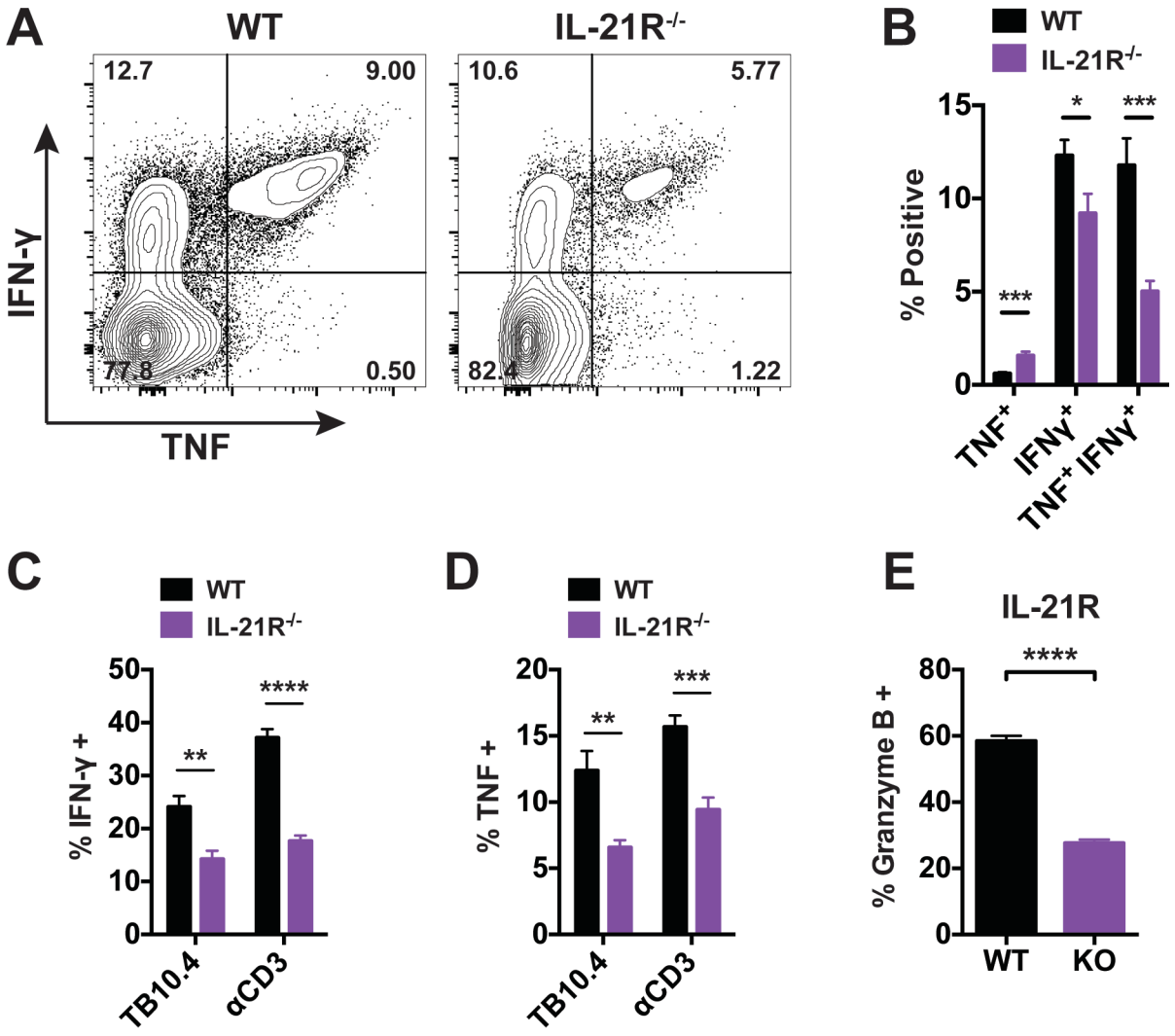

Figure 3-5. IL-21R$^{-/-}$ CD8^{+} T cells produce less IFN-γ, TNF, and granzyme B.

(A) Representative flow cytometry plots of lung CD8^{+} T cells of the indicated genotype 4 weeks after infection. Lung homogenates were stimulated *ex vivo* with TB10.4 peptide prior to IFN-γ and TNF ICS. (B) The percentage of WT and KO CD8^{+} T cells positive for the indicated combinations of IFN-γ and TNF as determined by ICS. (C,D) The total percentage of IFN-γ^{+} (C) and TNF^{+} (D) CD8^{+} T cells of the indicated genotype following *ex vivo* TB10.4 or αCD3/αCD28 stimulation. (E) The percentage of WT and KO CD8^{+} T cells positive for intracellular granzyme B staining in the lungs. Each bar represents the mean ± SEM (n = 9 mice per group) *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (paired Student's t-test). Data are representative of three independent experiments.

with TCR engagement and costimulation (72). However, IL-21 has never been implicated in $CD8^+$ T cell priming *in vivo*. To analyze priming, we generated retrogenic (Rg) $CD8^+$ T cells specific for the immunodominant antigen $TB10.4_{4\text{-}11}$ on both WT and IL-$21R^{-/-}$ backgrounds. Equal numbers of congenically marked WT and IL-$21R^{-/-}$ Rg cells were transferred into recipient mice 7 days after low-dose aerosol infection, and the ratio of WT and KO Rg $CD8^+$ T cells was assessed at days 8, 11, 13, and 15 in the lymph node. At the onset of priming, IL-$21R^{-/-}$ Rg cells expanded comparably to WT cells. The ratio of WT and IL-$21R^{-/-}$ Rg cells remained consistent from days 8 to 11 as the number of both WT and KO cells increased by over five fold (*Figure 3-6A and 3-6B*). The cells were labeled with a proliferation dye prior to transfer and both populations diluted the dye to the same degree by day 11, indicating that IL-$21R^{-/-}$ Rg cells were dividing comparably to WT Rg cells (*Figure 3-6C*).

Despite initially expanding well, IL-$21R^{-/-}$ Rg cells started to lag behind WT Rg cells by day 13 and were underrepresented in the lymph node through day 15 (*Figure 3-6A and 3-6B*). This pattern was mirrored in the lungs where the percentage and number of IL-$21R^{-/-}$ Rg cells began to decline after day 13 (*Figure 3-7A and 3-7B*). We tracked the transferred Rg cells through day 28 in the lungs and observed that the IL-$21R^{-/-}$ cells continued to decline (*Figure 3-7A and 3-7B*). These observations suggest that the initial cell divisions following priming can still occur in the absence of IL-21 signaling, but that IL-21 is required soon after priming for the robust expansion of $CD8^+$ T cells. Furthermore, IL-21 is required in both the lymph node and the lungs to support expansion.

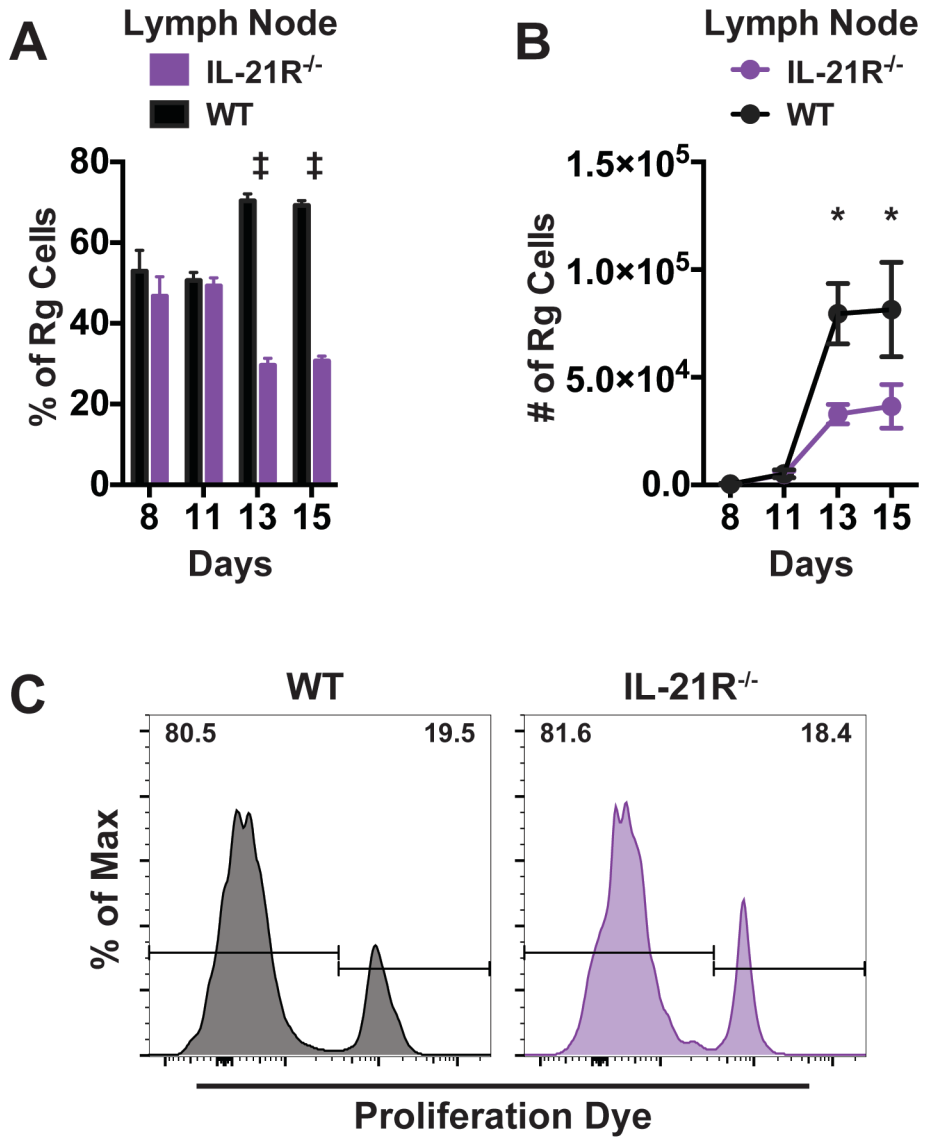

Figure 3-6. IL-21 supports the expansion of CD8$^+$ T cells in the lung draining lymph node.

Equal numbers of retrogenic (Rg) TB10.4-specific CD8$^+$ T cells were transferred into mice 7 days after low-dose aerosol infection with Mtb. (A) The percentage of total retrogenic (Rg) cells that were WT or IL-21R$^{-/-}$ in the mediastinal lymph node at days 8, 11, 13 and 15 following infection. (B) Total number of WT and IL-21R$^{-/-}$ Rg cells detected in the lymph node at the indicated time points. (C) Histograms depicting the dilution of the proliferation dye eflour 450 in Rg cells from the lymph node at day 11. Each group of samples (WT and IL-21R$^{-/-}$) was concatenated into a single histogram. Each bar or point represents the mean ± SEM (n = 4-5 mice per group) *P < 0.05, ***P < 0.001, ‡P < 0.0001 (Holm-Šídák multiple comparisons testing following two-way ANOVA). Data are from a single experiment.

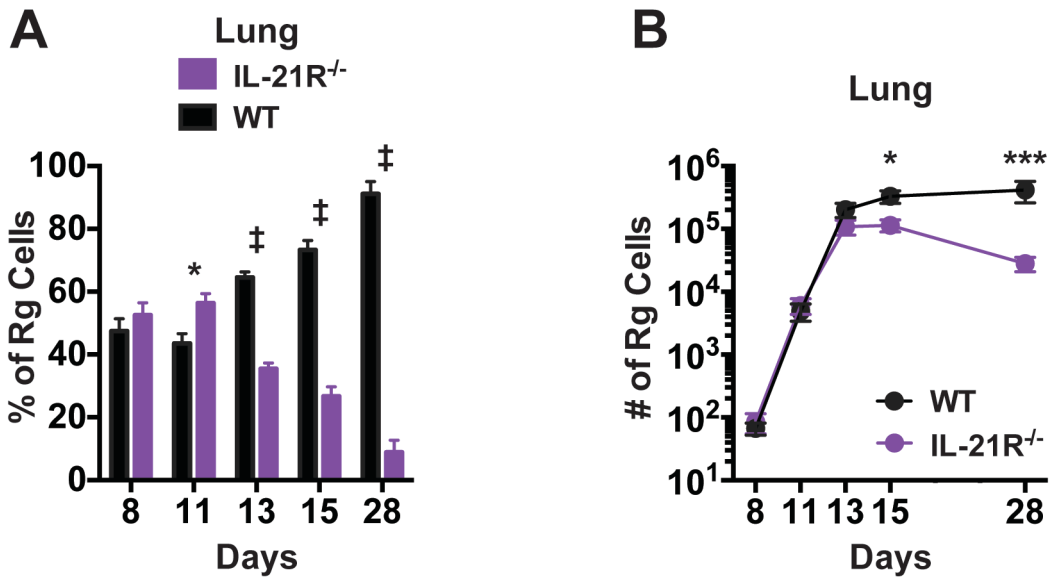

Figure 3-7. IL-21 is required for the continued expansion of CD8$^+$ T cells in the lungs during tuberculosis.

Equal numbers of retrogenic (Rg) TB10.4-specific CD8$^+$ T cells were transferred into mice 7 days after low-dose aerosol infection with Mtb. (A) The percentage of total retrogenic (Rg) cells that were WT or IL-21R$^{-/-}$ in the lungs at days 8, 11, 13, 15, and 28 following infection. (B) Total number of WT and IL-21R$^{-/-}$ Rg cells detected in the lungs at the indicated time points. Each bar or point represents the mean ± SEM (n = 4-5 mice per group) *P < 0.05, ***P < 0.001, ‡P < 0.0001 (Holm-Šídák multiple comparisons testing following two-way ANOVA). Data are from a single experiment.

IL-21R$^{-/-}$ mice have a diminished CD8^{+} T cell response throughout Mtb infection.

To date, no studies have characterized the susceptibility of IL-21R$^{-/-}$ mice to tuberculosis, and we are currently examining the immune response and bacterial burden in these mice. As an initial assessment, female IL-21R$^{-/-}$ mice and age-matched C57BL/6J controls were infected via the aerosol route and time points were performed at weeks four and sixteen. At week 4, IL-21R$^{-/-}$ mice had comparable percentages of CD4^{+} T cells and B cells in the lungs and spleens (data not show). Similar to our observations using the 1:1 mixed bone marrow chimeras, the overall number of CD8^{+} T cells was severely reduced in the lungs of IL-21R$^{-/-}$ mice, and this trend continued though week 16 (*Figure 3-8A*). In both WT and IL-21R$^{-/-}$ mice, approximately 30% of CD8^{+} T cells were specific for TB10.4$_{4-11}$ at week 4; however, TB10.4$_{4-11}$-specific cells were reduced due to the overall defect in CD8^{+} T expansion (*Figure 3-8B and data not shown*). IL-21R$^{-/-}$ mice had comparable numbers of CD8^{+} T cells in the spleen at week 4, but IL-21R$^{-/-}$ CD8^{+} T numbers declined as the infection progressed (*Figure 3-8C and 3-8D*). These observations confirm a substantial role for IL-21 in maintaining CD8^{+} T cell numbers during tuberculosis.

Loss of IL-21R increases CD8^{+} T cell exhaustion.

During chronic viral infections, T cell exhaustion is partially related to the upregulation of a number of inhibitory receptors, including PD-1 and TIM-3 (90,91). IL-21 is implicated in preventing CD8^{+} T cell exhaustion during LCMV infection (34,78,79), so we examined these exhaustion markers on TB10.4$_{4-11}$-specific CD8^{+} T cells. At week 16, the majority of WT TB10.4$_{4-11}$-specific CD8^{+} T cells expressed high levels of Tim-3,

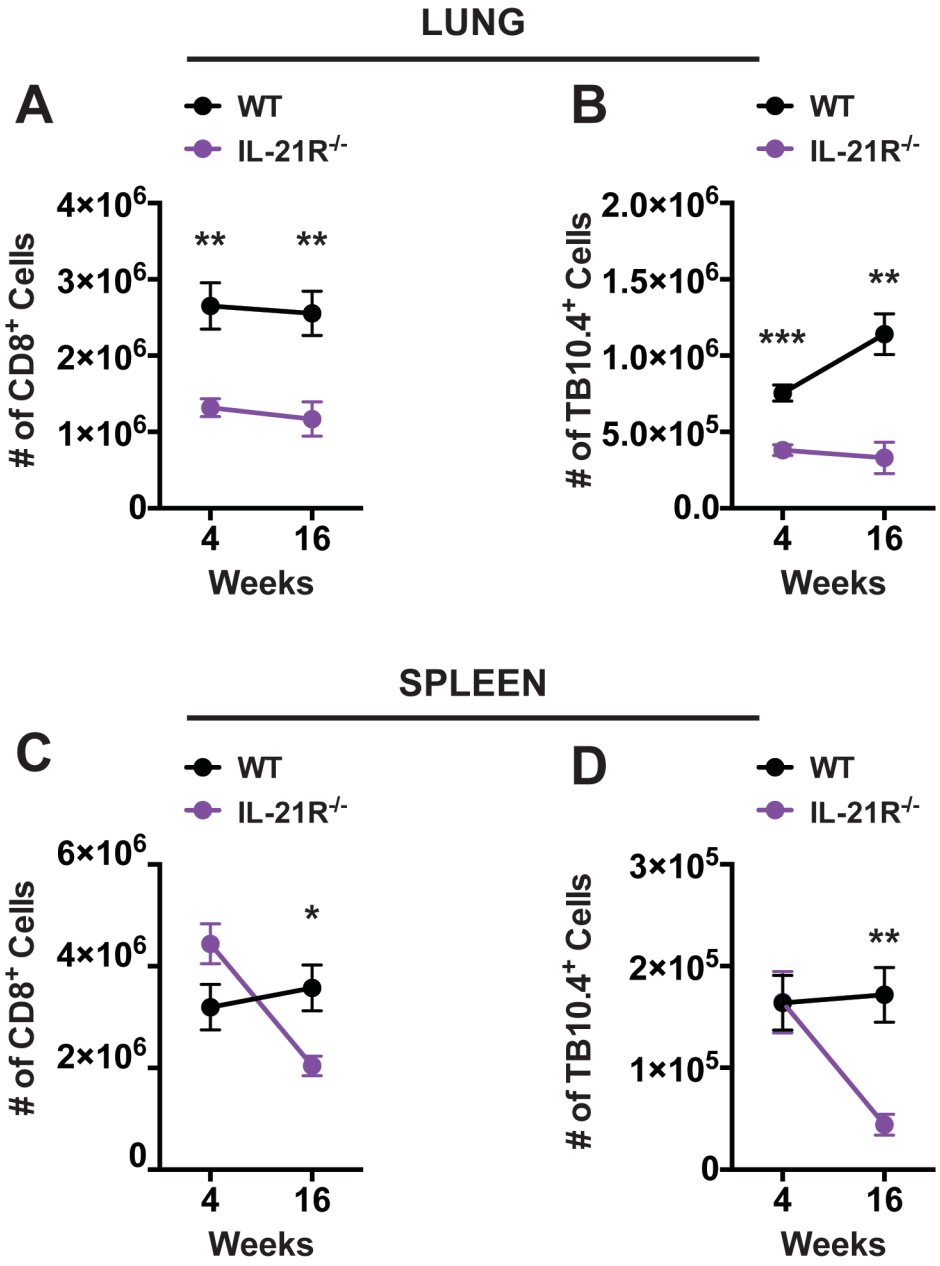

Figure 3-8. IL-21R$^{-/-}$ mice have a diminished CD8^{+} T cell response following Mtb infection.

WT and IL-21R$^{-/-}$ mice were infected via the aerosol route and total CD8^{+} T cells enumerated in the lungs (A) and spleens (C) at 4 and 16 weeks post infection. The number of TB10.4-specific cells was also determined in the lungs (B) and spleen (D) based on tetramer staining. Each point represents the mean ± SEM (n = 5 mice per group) *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Student's t-test). Data are from a single experiment.

but relatively low amounts of PD-1 (*Figure 3-9A*). TIM-3 expression was comparable between IL-21R$^{-/-}$ and WT cells but nearly all TIM-3^{+} IL-21R$^{-/-}$ cells were also PD-1^{+} (*Figure 3-9A and 3-9B*). Indeed, PD-1 expression was substantially increased on IL-21R$^{-/-}$ CD8^{+} T cells by both percentage and intensity (*Figure 3-9C and 3-9D*). This dramatic increase in PD-1 expression suggests IL-21R$^{-/-}$ CD8^{+} T cells are exhausted and may be functioning poorly.

To functionally address exhaustion, we measured IFN-γ and TNF production by intracellular cytokine staining in lung cells stimulated *ex vivo* with the immunodominant TB10.4$_{4-11}$ peptide. IL-21R$^{-/-}$ CD8 T cells still produced significant amounts of cytokine in response to peptide stimulation; however, the percentage of cells producing both IFN-γ and TNF was reduced (*Figure 3-10A and 3-10B*). The total amount of IFN-γ production was comparable between the WT and IL-21R$^{-/-}$ groups and the defect in double positive cells was the result of diminished TNF production (*Figure 3-10C and 3-10D*). TNF productions is typically one of first effector functions lost as CD8^{+} T cell become exhausted, while IFN-γ production persists longer (92). Thus, this observation is in line with research done in other chronic infections. A decrease in granzyme B levels was also observed in IL-21R$^{-/-}$ CD8^{+} T cells, suggesting multiple effector functions are compromised in the absence of IL-21 (*Figure 3-10E*). It appears that the absence of IL-21 leads to the accelerated exhaustion of CD8^{+} T cells during tuberculosis.

IL-21R$^{-/-}$ mice are more susceptible to chronic Mtb infection.

Depleting CD8^{+} T cells during tuberculosis only results in a survival defect after 200 days of infection (93). Thus, a defect in CD8^{+} T cell function alone is not expected

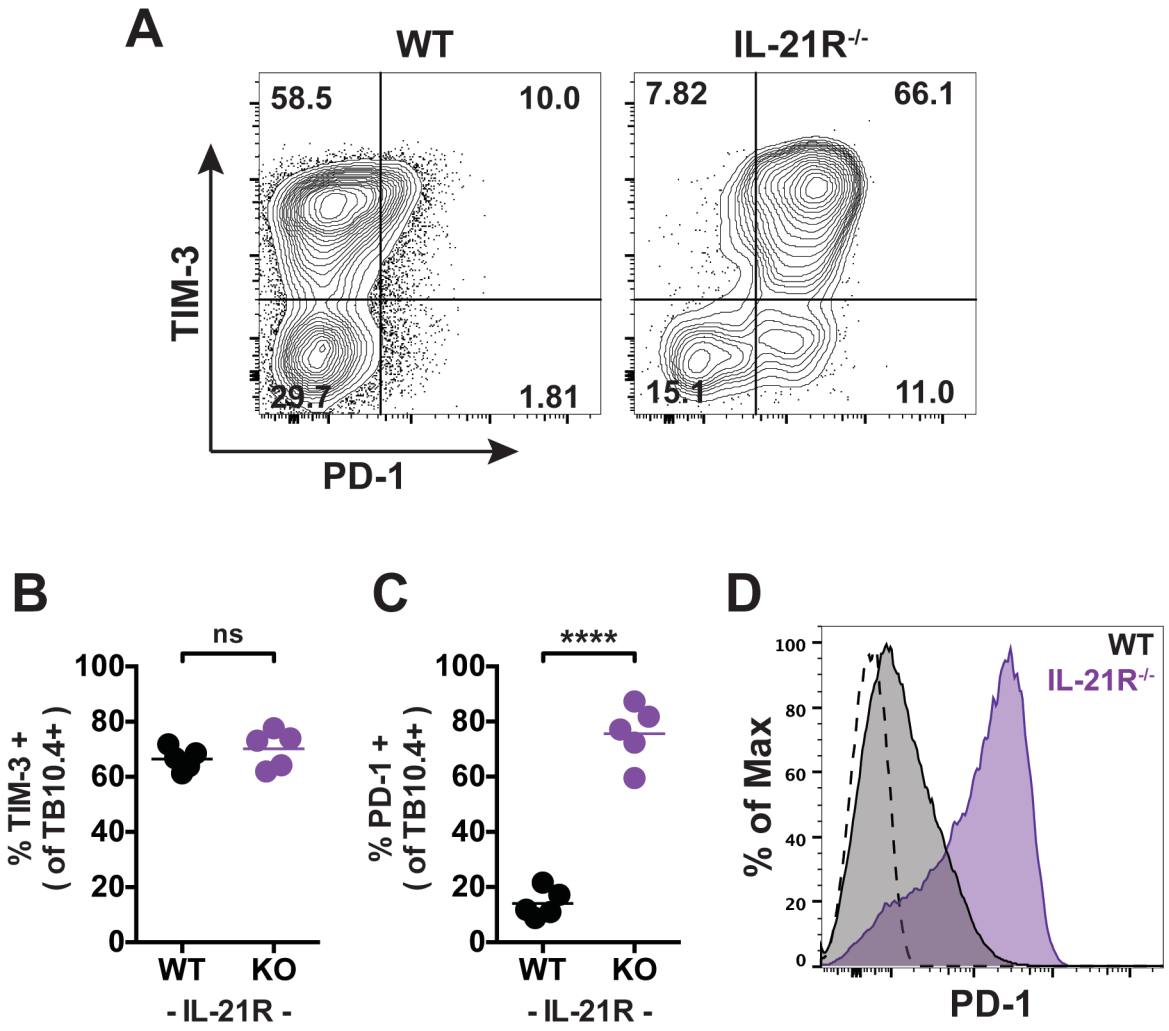

Figure 3-9. IL-21R$^{-/-}$ CD8^{+} T cells express high levels of PD-1 by 16 weeks post-infection.

(A) Representative flow cytometry plots of lung CD8^{+} TB10.4^{+} T cells of the indicated genotype 16 week post-infection. (B) The percentage of WT or IL-21R$^{-/-}$ CD8^{+} TB10.4^{+} T cells positive for TIM-3 expression in the lungs. (C) The percentage of WT or IL-21R$^{-/-}$ CD8^{+} TB10.4^{+} T cells positive for PD-1 expression in the lungs. (D) Histograms depicting PD-1 levels on lung CD8^{+} TB10.4^{+} T cells. Each group of samples (WT and IL-21R$^{-/-}$) was concatenated into a single histogram. Each bar represents the mean ± SEM (n = 5 mice per group) ****P < 0.0001 (unpaired Student's t-test). Data are from a single experiment.

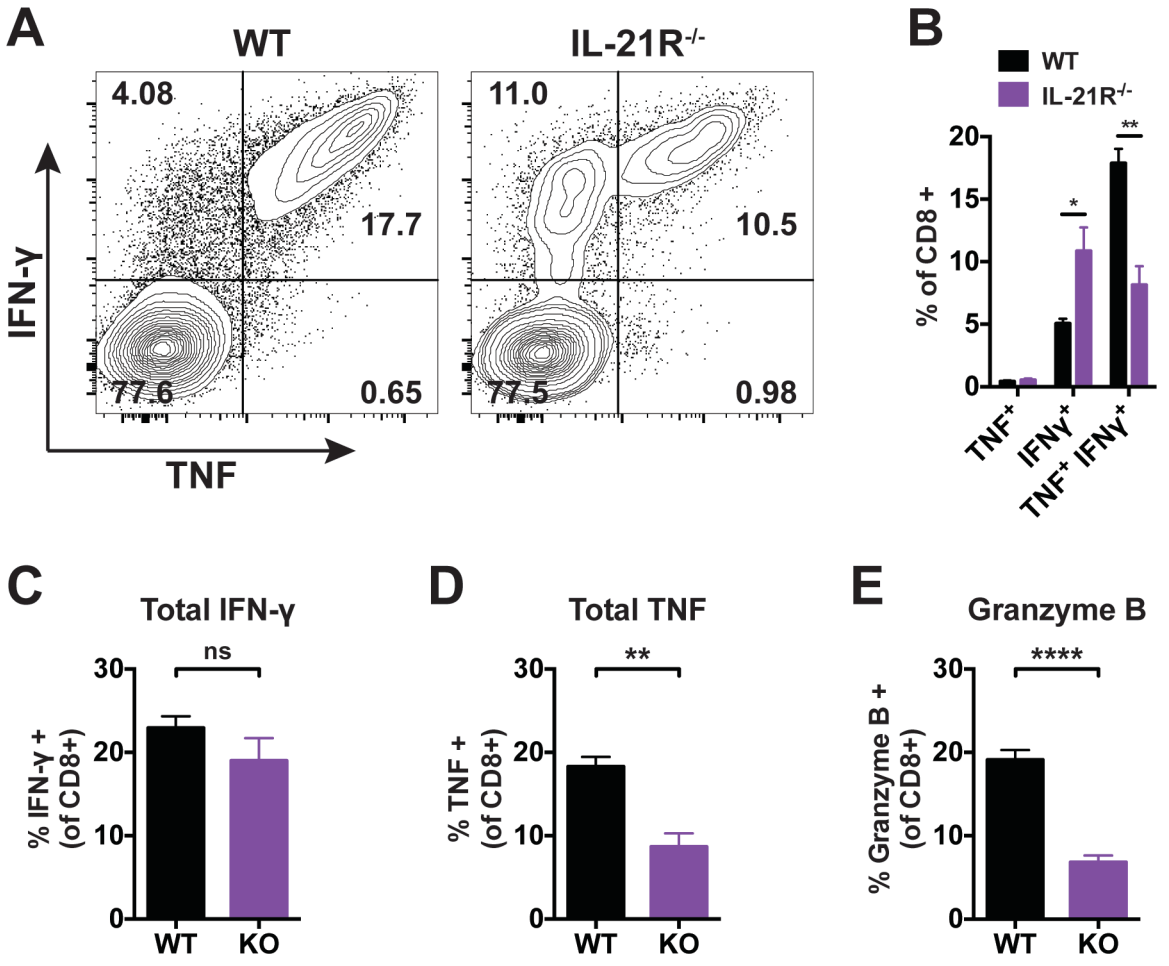

Figure 3-10. IL-21R$^{-/-}$ CD8^{+} T cells maintain IFN-γ production 16 weeks post-infection but produce less TNF.

(A) Representative flow cytometry plots of lung CD8^{+} T cells of the indicated genotype 16 weeks after infection. Lung homogenates were stimulated *ex vivo* with TB10.4 peptide prior to IFN-γ and TNF ICS. (B) The percentage of WT and KO CD8^{+} T cells positive for the indicated combinations of IFN-γ and TNF as determined by ICS. Here, IFN-γ^{+} and TNF^{+} indicates the percentage of the population producing only that cytokine. The total percentage of IFN-γ^{+} (C) and TNF^{+} (D) CD8^{+} T cells of the indicated genotype following *ex vivo* TB10.4 peptide stimulation. (E) The percentage of WT and KO CD8^{+} T cells positive for intracellular granzyme B staining in the lungs. Each bar represents the mean ± SEM (n = 9 mice per group) **P < 0.01, ***P < 0.001, ****P < 0.0001 (unpaired Student's t-test). Data are from a single experiment.

to cause an acute increase in susceptibility. For this reason, a potential survival defect in the IL-21R$^{-/-}$ mice is predicted to take a considerable amount of time to become evident. Consistent with this hypothesis, IL-21R$^{-/-}$ mice have survived past 125 days of infection without showing overt signs of illness (data not shown), and survival experiments are currently ongoing. At week four, bacterial burden was comparable between WT and IL-21R$^{-/-}$ mice in both the lungs and spleen (*Figure 3-11A and 3-11B*). This finding was expected, because the only observed defect in the immune response was a reduction in CD8^{+} T cells. By week 16, bacterial burden increased in the lungs of IL-21R$^{-/-}$ mice, indicating that they eventually fail to restrict bacterial growth (*Figure 3-11A*). Bacterial burden in the spleens remained comparable between the WT and KO groups at all time points (*Figure 3-11B*).

It is tempting to speculate that the dysfunctional CD8^{+} T cell response was contributing to this loss of bacterial control in IL-21R$^{-/-}$ mice; however, we observed additional alterations in the immune response by week 16. Though the amount of CD4^{+} T cells was unaltered at week 4, IL-21R$^{-/-}$ mice had reduced CD4^{+} T cell numbers by week 16 of infection (*Figure 3-11C*). Additionally, these mice had slightly increased number of neutrophils in the lungs (*Figure 3-11D*), though this may be a result of the increased bacterial burden. CD4^{+} T cells from IL-21R$^{-/-}$ mice produced comparable amounts of TNF and IFN-γ when stimulated *ex vivo* with ESAT6 peptide or anti-CD3/anti-CD28 (*Figure 3-12* and data not shown). This suggests that the remaining CD4^{+} T cells were functional and capable of responding to antigen. Based on the current data, we cannot know if CD4^{+} T cell functions are comprised in IL-21R$^{-/-}$ mice, but it is clear that CD4^{+} T cell numbers decline by week 16 of infection. For this reason,

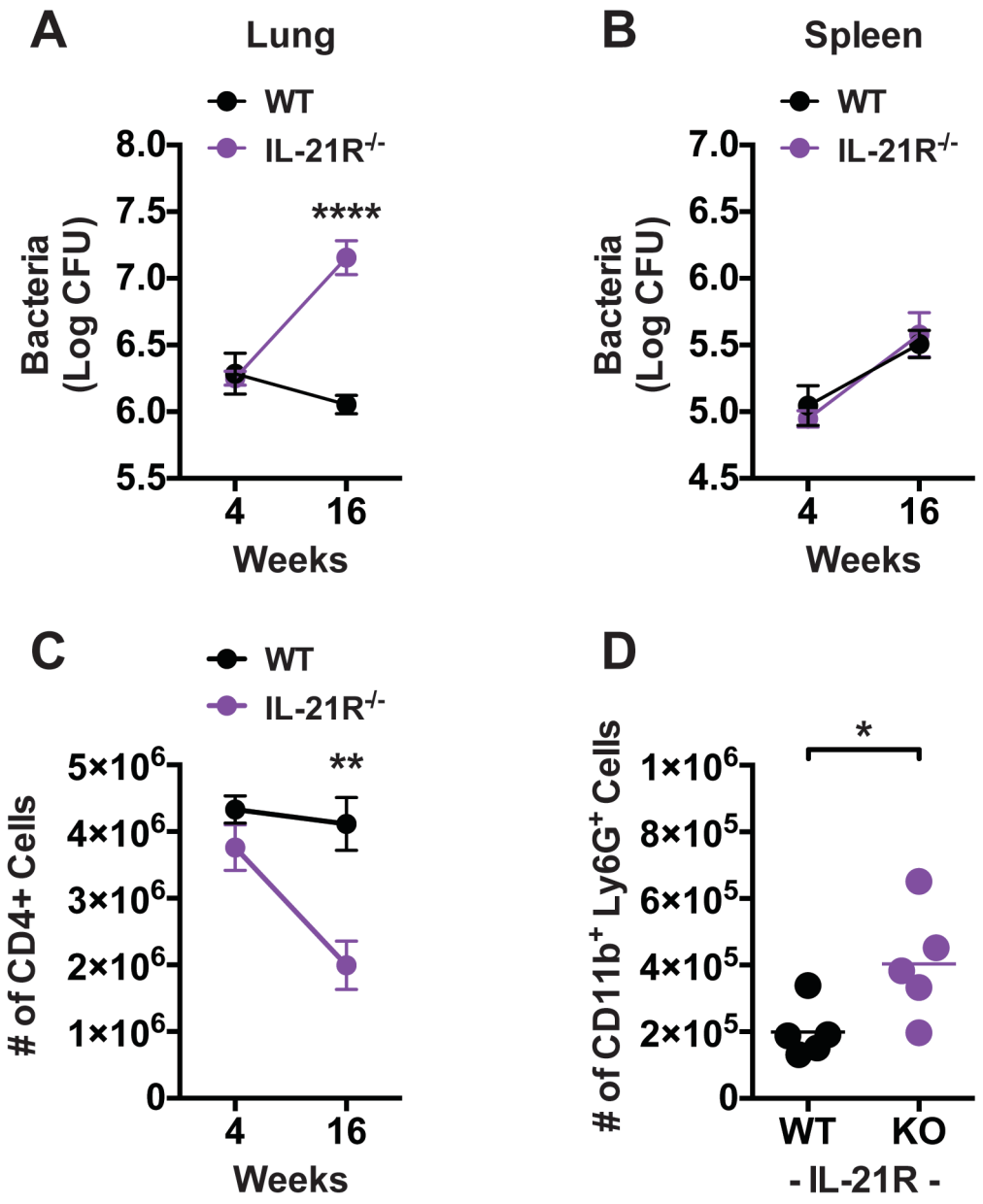

Figure 3-11. IL-21R$^{-/-}$ mice fail to control bacterial growth by week 16 and have diminished CD4^{+} T cell numbers.

Bacterial burden in the lungs (A) and spleens (B) at 4 and 16 weeks post infection. (C) The total number of CD4^{+} T cells was determined in the lungs at weeks 4 and 16 post infection. (D). Total number of neutrophils (CD11b^{+} Ly6G^{+}) in the lungs of infected mice at week 16. Each point with error bars represents the mean ± SEM. The points in (D) represent individual mice and the bars represents the mean. (n = 5 mice per group) *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (unpaired Student's t-test). Data are from a single experiment.

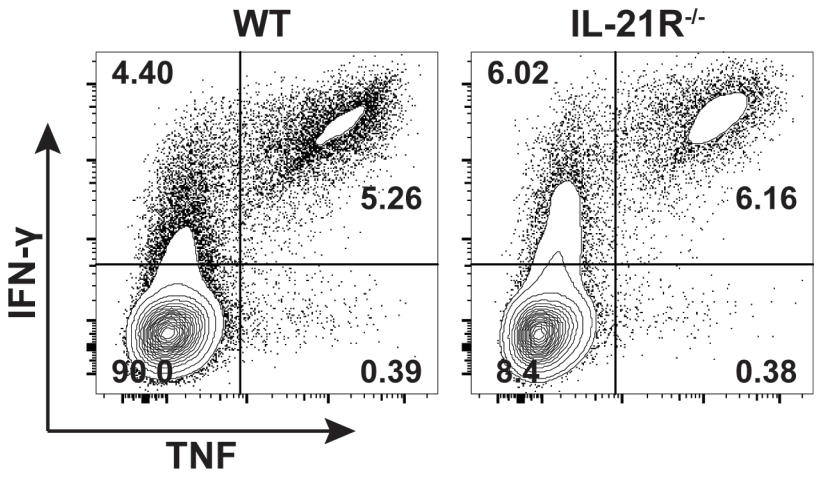

Figure 3-12. IL-21R$^{-/-}$ CD4^{+} T cell cytokine production is unaltered.

Representative flow cytometry plots gated on lung CD4^{+} T cells of the indicated genotype 16 weeks after infection. ICS was performed for IFN-γ and TNF production by CD4^{+} T cells from lung homogenate stimulated *ex vivo* with ESAT6 peptide. Shown are representative plots from a group of 5 mice. Data are from a single experiment.

it is likely that the combined reduction of $CD4^+$ and $CD8^+$ T cells is contributing to increased bacterial burden. Overall, IL-21 appears to regulate host resistance during chronic infection with Mtb.

Discussion

During chronic viral infections, IL-21 is a major mediator of $CD4^+$ T cell help and is essential to maintain a functional $CD8^+$ T response. This cytokine not only supports $CD8^+$ T cell numbers but also preserves cytokine production and prevents T cell exhaustion. Here, we demonstrate IL-21 is essential for the $CD8^+$ T cell response to Mtb. Without this cytokine, $CD8^+$ T cell numbers are dramatically reduced following aerosol infection and the remaining $CD8^+$ T cell population eventually adopts an exhausted phenotype. Furthermore, IL-$21R^{-/-}$ mice ultimately fail to control bacterial growth; indicating IL-21 is a mediator of resistance during chronic infection.

Using 1:1 mixed bone marrow chimeras, we demonstrate IL-21R is directly required on $CD8^+$ T cells during tuberculosis. This system offers the key advantage of directly comparing WT and IL-$21R^{-/-}$ $CD8^+$ T cells in the same inflammatory environment *in vivo*. Four week following aerosol infection, IL-$21R^{-/-}$ $CD8^+$ T cells are dramatically reduced in the blood and lungs relative to WT cells. Additionally, IL-$21R^{-/-}$ $CD8^+$ T cells produce less IFN-γ, TNF, and granzyme B. These effects resemble experiments with IL-$12R^{-/-}$ $CD8^+$ T cells (Chapter 2). IL-12 is well characterized as an essential cytokine for $CD4^+$ T cell responses during tuberculosis, and our data indicate it is also essential for $CD8^+$ T cells. Together IL-12 and IL-21 are significant drivers of the $CD8^+$ T cell response to Mtb.

Using the adoptive transfer of retrogenic Ag-specific $CD8^+$ T cells, we tracked the priming of IL-$21R^{-/-}$ cells in the lung-draining lymph node following low-dose aerosol infection. Initially, IL-$21R^{-/-}$ $CD8^+$ T cells respond comparably to WT cells and dilute their proliferation dye. However, IL-$21R^{-/-}$ cells lag behind WT cells as soon as 13 days post-

infection. Because IL-21 is required in the lymph node, we argue that it is a signal 3 cytokine and facilitates priming. Our data indicate IL-21 is dispensable for the initial cell divisions following priming but is soon required to promote expansion. Similar observations were made with type 1 IFN and IL-12 during other infections, and current data suggests signal 3 cytokines support CD8 accumulation by maintaining cell cycle progression several days after priming (94-96). Typically, APCs produce signal 3 cytokines, making IL-21 unique. Because Ag-specific CD4+ T cells are the likely source IL-21, these findings have implications for the coordinated priming of naïve $CD4^+$ and $CD8^+$ T cells during infection.

Similar to the findings in the lymph node, IL-$21R^{-/-}$ $CD8^+$ T cells initially expand well in the lungs but begin to recede in number by day 15. From days 15 to 28, the number of IL-$21R^{-/-}$ $CD8^+$ T cells continues to decline, suggesting an increase in cell death. IL-21 has been associated both with promoting $CD8^+$ T cell division and cell survival, depending on the pathogen (34,83). Based on the current data, we cannot address the roles of cell death versus continued proliferation in maintaining cell number in the lungs. Future experiments will be designed to address these possibilities.

In addition to examining the effects of IL-21 on $CD8^+$ T cells, we characterized the susceptibility of IL-$21R^{-/-}$ mice to tuberculosis. Four weeks following aerosol infection, IL-$21R^{-/-}$ mice had normal amounts of B and $CD4^+$ T cells, but severely reduced numbers of $CD8^+$ T cells. This observation supports our findings in the 1:1 mixed bone marrow chimeras and suggests IL-21 acts primarily on $CD8^+$ T cells at early time points. At sixteen weeks post infection, $CD8^+$ T cell numbers remain diminished and cells have increased expression of the inhibitory receptor PD-1. This was

accompanied by deceased production of TNF and granzyme B, indicating $CD8^+$ T cells were exhausted in the absence of IL-21 signaling. $CD8^+$ T cell exhaustion is common during chronic infections, but the role of exhaustion during tuberculosis is not clearly understood (90). Higher frequencies of PD-1^+ T cells are observed in patients with active pulmonary tuberculosis and PD-1 blockade *in vitro* can enhance Mtb-specific IFN-γ production (97). Thus, it is possible T cell exhaustion limits bacterial killing during chronic tuberculosis. Currently, we have no data on the kinetics of IL-21 production in the lungs of WT mice infected with Mtb. It will be interesting to determine if IL-21 levels remain consistent throughout disease and potentially correlate IL-21 concentrations with $CD8^+$ T cell exhaustion.

By week sixteen, IL-$21R^{-/-}$ mice fail to control bacterial growth in the lungs. This observation conflicts with a previous report examining bacterial burden in IL-$21^{-/-}$ mice (89). This study failed to identify a difference in bacterial control between WT and IL-$21^{-/-}$ mice by day 200 post-infection. There are a variety of differences in experimental approaches that could account for this discrepancy. These include the bacterial strains used, initial inoculum, housing conditions, and differences in the genetic background of the IL-$21R^{-/-}$ and IL-$21^{-/-}$ mice. Regardless, IL-$21R^{-/-}$ mice have a clear phenotype by week 16 in our facility.

It is possible that reduced $CD8^+$ T cell numbers and increased exhaustion are contributing to increased bacterial burden, but we also noted a sharp decline in $CD4^+$ T cells at week 16. IL-21 can direct the differentiation of T_H17 and Tfh cells, though it is not considered essential for their generation *in vivo*. Still, studies of chronic *T. gondii* infection indicate a decline in Tfh cell at later time points, suggesting IL-21 maintains

this population of $CD4^+$ T cells in some settings (83). During tuberculosis, Tfh-like $CD4^+CXCR5^+$ cells are associated with ectopic lymphoid structures in the lungs that contain well-organized B cell follicles (89). The precise role of these pulmonary B cell follicles is unclear, but they are associated with better control of bacterial growth in non-human primate models. Specifically, $CD4^+CXCR5^+$ Tfh-like cells preferentially migrate to these areas during infection and activate infected macrophages. IL-21 was even demonstrated to promote the formation of these pulmonary B cell follicles during murine tuberculosis (89). Thus, one hypothesis for the increased susceptibility of IL-$21R^{-/-}$ is a decrease in pulmonary B cell follicle formation concomitant with reduced Tfh cells. This could result in disorganized lung lesions and reduced bacterial killing. This is obviously one of many conceivable explanations, and future studies will address these possibilities in IL-$21R^{-/-}$ mice.

We have uncovered an essential role for IL-21 during tuberculosis. Our data indicate it is a necessity for $CD8^+$ T cells and has other effects on immunity during chronic disease. We believe IL-21 has promise as a therapeutic intervention and has the potential to augment vaccine efficacy. Future experiments must be designed to test these exciting possibilities.

Materials and methods

Mice

C57BL/6 (WT), CD45.1 (B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ), CD90.1 (B6.PL-Thy1^{a}/CyJ), and IL-21R$^{-/-}$ (B6N.129-Il21r^{tm1Kopf}/J) (98) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were 6 to 10 weeks old at the start of all experiments. All animal experiments were performed in accordance with National and European Commission guidelines for the care and handling of laboratory animals and were approved by the National Veterinary Directorate and by the local Animal Ethical Committee or Institutional Animal Care and Use Committee (Animal Welfare Assurance no. A3023-01 [DFCI] or A3306-01 [UMMS]), under Public Health Service assurance of Office of Laboratory Animal Welfare guidelines). Mice infected with *M. tuberculosis* were housed in a biosafety level 3 facility under specific pathogen-free conditions at DFCI or at UMMS.

Generation of mouse bone marrow chimeras

1:1 mixed bone barrow chimeras were made by lethally irradiating CD90.1^{+} recipients (2 doses of 600 rads separated by three hours). Bone marrow was flushed from the femurs, tibia, and humeri of donor mice and RBC lysed. Bone marrow cells were then enumerated and groups were combined in a 1:1 ratio. Each recipient mouse received a total of 10^{7} bone marrow cells (5×10^{6} of WT and 5×10^{6} of KO) via lateral tail vein injection and was kept on antibiotic-treated water for 5 weeks following irradiation. Mice were checked for reconstitution by retro-orbital bleeding to assess the ratio of

donor cells in the peripheral blood by flow cytometry. Bone marrow chimeras were infected with Mtb 8-10 weeks after transfer of the bone marrow cells.

Generation of retrogenic mice

TCR retroviral constructs were generated as 2A-linked single open reading frames using PCR and cloned into a murine stem cell virus-based retroviral vector with a GFP marker as previously described (99). Details of cloning strategies and primer sequences are available upon request (Samuel.behar@umassmed.edu). Retroviral-mediated stem cell gene transfer was performed as previously described (99).

Experimental infection and bacterial quantification

Infection with *M. tuberculosis* (Erdman strain) was performed via the aerosol route, and mice received a day 1 inoculum of 50-200 CFU. A bacterial aliquot was thawed, sonicated twice for 10 seconds in a cup horn sonicator, and then diluted in 0.9% NaCl–0.02% Tween 80. A 15 ml suspension of *M. tuberculosis* was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technologies) and mice were infected using a nose-only exposure unit (Intox Products). Alternatively, the bacterial aliquot was diluted in a final volume of 5ml, and mice were infected using a Glas-Col aerosol-generation device. At different times post-infection, mice were euthanized by carbon dioxide inhalation, organs were aseptically removed, individually homogenized and viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H11 agar plates. Plates were incubated at 37ºC and *M. tuberculosis* colonies were counted after 21 days.

FACS analysis

Cell suspensions from lung, spleen and lymph nodes were prepared by gentle disruption of the organs through a 70µm nylon strainer (Fisher) or using the GentleMacs apparatus (Miltenyi Biotec, Germany) according to the manufacturer's instructions. For lung preparations, tissue was digested for 30-60 min at 37°C in cRPMI with 300U/ml collagenase (Sigma) prior to straining. Erythrocytes were lysed using a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM sodium EDTA pH 7.2) and, after washing, cells were resuspended in supplemented RPMI (cRPMI - 10% heat inactivated FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml streptomycin and 50 U/ml penicillin, all from Invitrogen) or MACS buffer (Miltenyi Biotec, Germany). Cells were enumerated in 4% trypan blue on a hemocytometer or using a MACSQuant flow cytometer (Miltenyi Biotec, Germany). Surface staining was performed with antibodies specific for mouse CD3 (clone 17A2), CD3ε (clone 145-2C11), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD19 (clone 6D5), CD44 (clone IM7), CD62L (clone MEL-14), CD45.1 (clone A20), CD45.2 (clone 104), CD90.1 (clone OX-7), CD90.2 (clone 53-2.1), CD127 (clone A7R34), KLRG1 (clone 2F1/KLRG1) and Vα2 (clone B20.1), (from Biolegend, CA, USA, or from BD Pharmingen, CA, USA). The tetramers of TB10.4_{4-11}-loaded H-2 K^b were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA, USA). All staining was performed for 20 min at 4°C and, unless otherwise stated, cells were fixed before acquisition with 1% formaldehyde in PBS for 30-60 min. Cell analysis was performed on a FACS Canto (Becton Dickinson, NJ, USA) or on a MACSQuant flow

cytometer (Miltenyi Biotec, Germany). Data were analyzed using FlowJo Software (Tree Star, OR, USA). For FACS analysis, single-lymphocyte events were gated by forward scatter versus height and side scatter for size and granularity.

Intracellular cytokine staining

5×10^5-1×10^6 cells were plated in each well of a round bottom 96-well plate and incubated in the presence of TB10.$4_{4\text{-}11}$ peptide (10 μM; New England Peptide). Incubation in the presence of αCD3/αCD28 (1 μg/mL; BioLegend) or in the absence of stimuli were used as positive and negative controls, respectively. Cells were incubated for 1 hours at 37ºC, at which point GolgiPlug solution (BD Pharmingen, CA, USA) was added to each well for the remaining 4 hours. Cells were collected after the 5 hours stimulation and then surface stained with the antibodies described above, followed by intracellular staining for IFN-γ (clone XMG1.2), TNF (clone MPX6-T22), or granzyme B (clone gb11) using the BD Permwash Kit (BD Pharmingen, CA, USA) as per manufacturer's instructions.

Adoptive T cell transfer

Single cell suspensions of pools of spleens and lymph nodes from naive retrogenic mice (6 to 12 weeks post reconstitution) were prepared. $CD8^+$ T cells were purified from each suspension using the $CD8^+$ T cell isolation kit and magnetic separation (STEMCELL Technologies Inc, Canada). After purification, cells were counted and transferred via the tail vein into congenically marked recipients (CD45.1 or CD90.1), which had been infected 7 days earlier with virulent Mtb (Erdman) via the

aerosol route. For all experiments, 3×10^4 -5×10^4 cells of each group were transferred into each recipient.

Measurement of cell proliferation

For analysis of cell proliferation of retrogenic cells after adoptive transfer, bead-purified naïve Rg cells (see above) were labeled with 5 μM cell proliferation dye efluor 450 (eBiosciences) in PBS for 20 min at room temperature, followed by extensive washing.

Cell isolation and microarray analysis

Female C57BL/6 mice were infected with Mtb Erdman as described above. At the indicated time points, mice were euthanized by cervical dislocation and lungs were harvested after perfusion with collagenase containing media. Lungs were allowed to digest in collagenase-containing media for 15 minutes before being homogenized into single cell suspensions. At this point, the lungs from 3 individual mice were combined into a single sample. T cells were then purified by negative magnetic bead selection (Miltenyi Biotec, Germany). Purified cells were stained to distinguish $CD4^+$ and $CD8^+$ T cells (CD19, CD3, CD4, CD8). For cell sorting, stained cells were suspended in MACS buffer (Miltenyi Biotec, Germany) and deposited in collection tubes using a BD Canto flow cytometer (Becton Dickinson, NJ, USA). 50,000 $CD19^-CD3^+CD4^+$ or $CD19^-CD3^+CD8^+$ cells were sorted directly into TRIzol Reagent (Life Technologies, California) and immediately frozen. RNA extraction, microarray hybridization (Affymetrix MoGene 1.0ST array) and data processing were done at the ImmGen Project processing center.

Details of the data analysis and quality control can be found at (www.immgen.org).

Statistical analysis

All data are represented as mean with SEM. Comparisons of two groups within 1:1 mixed bone marrow chimeras were done with a paired student's t-test. All other comparisons were done with an unpaired student's t-test and are indicated in the figure legends. Comparisons of more than two groups were done using Holm-Šídák multiple comparisons testing following two-way ANOVA. Significance was represented by the following symbols: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and ‡P < 0.0001.

References

1. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. Nature Reviews Immunology. 2009 Jul;9(7):480–90.

2. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, et al. Cloning of the gamma chain of the human IL-2 receptor. Science. 1992 Jul 17;257(5068):379–82.

3. Noguchi M, Nakamura Y, Russell SM, Ziegler SF, Tsang M, Cao X, et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. Science. 1993 Dec 17;262(5141):1877–80.

4. Davis MR, Zhu Z, Hansen DM, Bai Q, Fang Y. The role of IL-21 in immunity and cancer. Cancer Lett. 2015 Mar 28;358(2):107–14.

5. Kündig TM, Schorle H, Bachmann MF, Hengartner H, Zinkernagel RM, Horak I. Immune responses in interleukin-2-deficient mice. Science. 1993 Nov 12;262(5136):1059–61.

6. Cousens LP, Orange JS, Biron CA. Endogenous IL-2 contributes to T cell expansion and IFN-gamma production during lymphocytic choriomeningitis virus infection. J Immunol. 1995 Dec 15;155(12):5690–9.

7. D'Souza WN, Lefrançois L. IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. J Immunol. 2003 Dec 1;171(11):5727–35.

8. Williams MA, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature. 2006 Jun 15;441(7095):890–3.

9. Bachmann MF, Wolint P, Walton S, Schwarz K, Oxenius A. Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections. Eur J Immunol. 2007 Jun;37(6):1502–12.

10. Obar JJ, Molloy MJ, Jellison ER, Stoklasek TA, Zhang W, Usherwood EJ, et al. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. Proceedings of the National Academy of Sciences. 2010 Jan 5;107(1):193–8.

11. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nature Reviews Immunology. 2012 Mar;12(3):180–90.

12. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. Nat Immunol. 2000 Nov;1(5):426–32.

13. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc Natl Acad Sci USA. 2001 Jul 17;98(15):8732–7.

14. Judge AD, Zhang X, Fujii H, Surh CD, Sprent J. Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. J Exp Med. 2002 Oct 7;196(7):935–46.

15. Tan JT, Ernst B, Kieper WC, LeRoy E, Sprent J, Surh CD. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. J Exp Med. 2002 Jun 17;195(12):1523–32.

16. Schluns KS, Williams K, Ma A, Zheng XX, Lefrançois L. Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. J Immunol. 2002 May 15;168(10):4827–31.

17. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, et al. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity. 2007 Aug;27(2):281–95.

18. Rubinstein MP, Lind NA, Purton JF, Filippou P, Best JA, McGhee PA, et al. IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response. Blood. 2008 Nov 1;112(9):3704–12.

19. Lefrançois L, Obar JJ. Once a killer, always a killer: from cytotoxic T cell to memory cell. Immunol Rev. 2010 May;235(1):206–18.

20. Ozaki K, Kikly K, Michalovich D, Young PR, Leonard WJ. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. Proc Natl Acad Sci USA. 2000 Oct 10;97(21):11439–44.

21. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature. 2000 Nov 2;408(6808):57–63.

22. Asao H, Okuyama C, Kumaki S, Ishii N, Tsuchiya S, Foster D, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. J Immunol. 2001 Jul 1;167(1):1–5.

23. Habib T, Senadheera S, Weinberg K, Kaushansky K. The common gamma chain (gamma c) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation via JAK3. Biochemistry. 2002 Jul 9;41(27):8725–31.

24. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu Rev Immunol. 2008;26:57–79.

25. Jin H, Carrio R, Yu A, Malek TR. Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. J Immunol. 2004 Jul 1;173(1):657–65.

26. Wu Z, Kim H-P, Xue H-H, Liu H, Zhao K, Leonard WJ. Interleukin-21 receptor gene induction in human T cells is mediated by T-cell receptor-induced Sp1 activity. Mol Cell Biol. 2005 Nov;25(22):9741–52.

27. Kim H-P, Korn LL, Gamero AM, Leonard WJ. Calcium-dependent activation of interleukin-21 gene expression in T cells. J Biol Chem. 2005 Jul 1;280(26):25291–7.

28. Brandt K, Bulfone-Paus S, Foster DC, Rückert R. Interleukin-21 inhibits dendritic cell activation and maturation. Blood. 2003 Dec 1;102(12):4090–8.

29. Distler JHW, Jüngel A, Kowal-Bielecka O, Michel BA, Gay RE, Sprott H, et al. Expression of interleukin-21 receptor in epidermis from patients with systemic sclerosis. Arthritis Rheum. 2005 Mar;52(3):856–64.

30. Caruso R, Fina D, Peluso I, Fantini MC, Tosti C, Del Vecchio Blanco G, et al. IL-21 is highly produced in Helicobacter pylori-infected gastric mucosa and promotes gelatinases synthesis. J Immunol. 2007 May 1;178(9):5957–65.

31. Coquet JM, Kyparissoudis K, Pellicci DG, Besra G, Berzins SP, Smyth MJ, et al. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. J Immunol. 2007 Mar 1;178(5):2827–34.

32. Harada M, Magara-Koyanagi K, Watarai H, Nagata Y, Ishii Y, Kojo S, et al. IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. J Exp Med. 2006 Dec 25;203(13):2929–37.

33. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KHG. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity. 2009 Aug 21;31(2):331–41.

34. Fröhlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. Science. 2009 Jun 19;324(5934):1576–80.

35. Ortega C, Fernández-A S, Carrillo JM, Romero P, Molina IJ, Moreno JC, et al. IL-17-producing CD8+ T lymphocytes from psoriasis skin plaques are cytotoxic effector cells that secrete Th17-related cytokines. Journal of Leukocyte Biology. 2009 Aug;86(2):435–43.

36. Williams LD, Bansal A, Sabbaj S, Heath SL, Song W, Tang J, et al. Interleukin-21-producing HIV-1-specific CD8 T cells are preferentially seen in elite controllers. Journal of Virology. 2011 Mar;85(5):2316–24.

37. Liu SM, King C. IL-21-producing Th cells in immunity and autoimmunity. The Journal of Immunology. 2013 Oct 1;191(7):3501–6.

38. Ozaki K, Spolski R, Feng CG, Qi C-F, Cheng J, Sher A, et al. A critical role for IL-21 in regulating immunoglobulin production. Science. 2002 Nov 22;298(5598):1630–4.

39. Ozaki K, Spolski R, Ettinger R, Kim H-P, Wang G, Qi C-F, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. J Immunol. 2004 Nov 1;173(9):5361–71.

40. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. J Immunol. 2007 Dec 15;179(12):8180–90.

41. Ettinger R, Sims GP, Fairhurst A-M, Robbins R, da Silva YS, Spolski R, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. J Immunol. 2005 Dec 15;175(12):7867–79.

42. Avery DT, Deenick EK, Ma CS, Suryani S, Simpson N, Chew GY, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. Journal of Experimental Medicine. 2010 Jan 18;207(1):155–71.

43. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. Journal of Experimental Medicine. 2010 Feb 15;207(2):365–78.

44. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. Journal of Experimental Medicine. 2010 Feb 15;207(2):353–63.

45. King IL, Mohrs K, Mohrs M. A nonredundant role for IL-21 receptor signaling in plasma cell differentiation and protective type 2 immunity against gastrointestinal helminth infection. The Journal of Immunology. 2010 Nov 15;185(10):6138–45.

46. Wurster AL, Rodgers VL, Satoskar AR, Whitters MJ, Young DA, Collins M, et al. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. J Exp Med. 2002 Oct 7;196(7):969–77.

47. Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF, Cheever AW, et al. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J Clin Invest. 2006 Jul;116(7):2044–55.

48. Fröhlich A, Marsland BJ, Sonderegger I, Kurrer M, Hodge MR, Harris NL, et al. IL-21 receptor signaling is integral to the development of Th2 effector responses in

vivo. Blood. 2007 Mar 1;109(5):2023–31.

49. Kastirr I, Maglie S, Paroni M, Alfen JS, Nizzoli G, Sugliano E, et al. IL-21 is a central memory T cell-associated cytokine that inhibits the generation of pathogenic Th1/17 effector cells. The Journal of Immunology. 2014 Oct 1;193(7):3322–31.

50. Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. J Immunol. 2004 Jul 1;173(1):68–78.

51. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature. 2007 Jul 26;448(7152):480–3.

52. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity. 2008 Jul 18;29(1):138–49.

53. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity. 2008 Jul 18;29(1):127–37.

54. Rasheed MAU, Latner DR, Aubert RD, Gourley T, Spolski R, Davis CW, et al. Interleukin-21 is a critical cytokine for the generation of virus-specific long-lived plasma cells. Journal of Virology. 2013 Jul;87(13):7737–46.

55. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. PLoS ONE. 2011;6(3):e17739.

56. Attridge K, Wang CJ, Wardzinski L, Kenefeck R, Chamberlain JL, Manzotti C, et al. IL-21 inhibits T cell IL-2 production and impairs Treg homeostasis. Blood. 2012 May 17;119(20):4656–64.

57. Schmitz I, Schneider C, Fröhlich A, Frebel H, Christ D, Leonard WJ, et al. IL-21 restricts virus-driven Treg cell expansion in chronic LCMV infection. PLoS Pathog. 2013;9(5):e1003362.

58. Coquet JM, Chakravarti S, Smyth MJ, Godfrey DI. Cutting edge: IL-21 is not essential for Th17 differentiation or experimental autoimmune encephalomyelitis. J Immunol. 2008 Jun 1;180(11):7097–101.

59. Sonderegger I, Kisielow J, Meier R, King C, Kopf M. IL-21 and IL-21R are not required for development of Th17 cells and autoimmunity in vivo. Eur J Immunol. 2008 Jul;38(7):1833–8.

60. Korn T, Bettelli E, Gao W, Awasthi A, Jäger A, Strom TB, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007 Jul 26;448(7152):484–7.

61. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of TH17 cells. Nature. 2008 Jun 19;453(7198):1051–7.

62. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol. 2007 Sep;8(9):967–74.

63. Mittal A, Murugaiyan G, Beynon V, Hu D, Weiner HL. IL-27 induction of IL-21 from human CD8+ T cells induces granzyme B in an autocrine manner. Immunology and Cell Biology. 2012 Sep;90(8):831–5.

64. Li Y, Yee C. IL-21 mediated Foxp3 suppression leads to enhanced generation of antigen-specific CD8+ cytotoxic T lymphocytes. Blood. 2008 Jan 1;111(1):229–35.

65. Kasaian MT, Whitters MJ, Carter LL, Lowe LD, Jussif JM, Deng B, et al. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. Immunity. 2002 Apr;16(4):559–69.

66. Ma H-L, Whitters MJ, Konz RF, Senices M, Young DA, Grusby MJ, et al. IL-21 activates both innate and adaptive immunity to generate potent antitumor responses that require perforin but are independent of IFN-gamma. J Immunol. 2003 Jul 15;171(2):608–15.

67. Zeng R, Spolski R, Finkelstein SE, Oh S, Kovanen PE, Hinrichs CS, et al. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. J Exp Med. 2005 Jan 3;201(1):139–48.

68. Li Y, Bleakley M, Yee C. IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response. J Immunol. 2005 Aug 15;175(4):2261–9.

69. White L, Krishnan S, Strbo N, Liu H, Kolber MA, Lichtenheld MG, et al. Differential effects of IL-21 and IL-15 on perforin expression, lysosomal degranulation, and proliferation in CD8 T cells of patients with human immunodeficiency virus-1 (HIV). Blood. 2007 May 1;109(9):3873–80.

70. Ostiguy V, Allard E-L, Marquis M, Leignadier J, Labrecque N. IL-21 promotes T lymphocyte survival by activating the phosphatidylinositol-3 kinase signaling cascade. Journal of Leukocyte Biology. 2007 Sep;82(3):645–56.

71. Novy P, Huang X, Leonard WJ, Yang Y. Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection. The

Journal of Immunology. 2011 Mar 1;186(5):2729–38.

72. Casey KA, Mescher MF. IL-21 promotes differentiation of naive CD8 T cells to a unique effector phenotype. J Immunol. 2007 Jun 15;178(12):7640–8.

73. Khattar M, Miyahara Y, Schroder PM, Xie A, Chen W, Stepkowski SM. Interleukin-21 is a critical regulator of CD4 and CD8 T cell survival during priming under Interleukin-2 deprivation conditions. PLoS ONE. 2014;9(1):e85882.

74. Yi JS, Ingram JT, Zajac AJ. IL-21 deficiency influences CD8 T cell quality and recall responses following an acute viral infection. The Journal of Immunology. 2010 Oct 15;185(8):4835–45.

75. Alves NL, Arosa FA, van Lier RAW. IL-21 sustains CD28 expression on IL-15-activated human naive CD8+ T cells. J Immunol. 2005 Jul 15;175(2):755–62.

76. Sutherland APR, Joller N, Michaud M, Liu SM, Kuchroo VK, Grusby MJ. IL-21 promotes CD8+ CTL activity via the transcription factor T-bet. The Journal of Immunology. 2013 Apr 15;190(8):3977–84.

77. Søndergaard H, Frederiksen KS, Thygesen P, Galsgaard ED, Skak K, Kristjansen PEG, et al. Interleukin 21 therapy increases the density of tumor infiltrating CD8+ T cells and inhibits the growth of syngeneic tumors. Cancer Immunol Immunother. 2007 Sep;56(9):1417–28.

78. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. Science. 2009 Jun 19;324(5934):1569–72.

79. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. Science. 2009 Jun 19;324(5934):1572–6.

80. Battegay M, Moskophidis D, Rahemtulla A, Hengartner H, Mak TW, Zinkernagel RM. Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. Journal of Virology. 1994 Jul;68(7):4700–4.

81. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. Journal of Virology. 1994 Dec;68(12):8056–63.

82. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med. 1998 Dec 21;188(12):2205–13.

83. Stumhofer JS, Silver JS, Hunter CA. IL-21 is required for optimal antibody production and T cell responses during chronic Toxoplasma gondii infection. PLoS ONE. 2013;8(5):e62889.

84. Ertelt JM, Johanns TM, Rowe JH, Way SS. Interleukin (IL)-21-independent

pathogen-specific CD8+ T-cell expansion, and IL-21-dependent suppression of CD4+ T-cell IL-17 production. Immunology. 2010 Oct;131(2):183–91.

85. Pérez-Mazliah D, Ng DHL, Freitas do Rosário AP, McLaughlin S, Mastelic-Gavillet B, Sodenkamp J, et al. Disruption of IL-21 Signaling Affects T Cell-B Cell Interactions and Abrogates Protective Humoral Immunity to Malaria. PLoS Pathog. 2015 Mar;11(3):e1004715.

86. Rahman S, Rehn A, Rahman J, Andersson J, Svensson M, Brighenti S. Pulmonary tuberculosis patients with a vitamin D deficiency demonstrate low local expression of the antimicrobial peptide LL-37 but enhanced FoxP3(+) regulatory T cells and IgG-secreting cells. Clin Immunol. 2015 Feb;156(2):85–97.

87. Pavan Kumar N, Anuradha R, Andrade BB, Suresh N, Ganesh R, Shankar J, et al. Circulating biomarkers of pulmonary and extrapulmonary tuberculosis in children. Clin Vaccine Immunol. 2013 May;20(5):704–11.

88. Kumar NP, Sridhar R, Hanna LE, Banurekha VV, Nutman TB, Babu S. Decreased frequencies of circulating CD4 T follicular helper cells associated with diminished plasma IL-21 in active pulmonary tuberculosis. PLoS ONE. 2014;9(10):e111098.

89. Slight SR, Rangel Moreno J, Gopal R, Lin Y, Fallert Junecko BA, Mehra S, et al. CXCR5 T helper cells mediate protective immunity against tuberculosis. J Clin Invest. 2013 Feb;123(2):712–26.

90. Behar SM, Carpenter SM, Booty MG, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. Seminars in Immunology. 2014 Dec;26(6):559–77.

91. Youngblood B, Wherry EJ, Ahmed R. Acquired transcriptional programming in functional and exhausted virus-specific CD8 T cells. Curr Opin HIV AIDS. 2012 Jan;7(1):50–7.

92. Wherry EJ. T cell exhaustion. Nat Immunol. 2011 Jun;12(6):492–9.

93. Mogues T, Goodrich ME, Ryan L, LaCourse R, North RJ. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. J Exp Med. 2001 Feb 5;193(3):271–80.

94. Aichele P, Unsoeld H, Koschella M, Schweier O, Kalinke U, Vucikuja S. CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion. J Immunol. 2006 Apr 15;176(8):4525–9.

95. Keppler SJ, Theil K, Vucikuja S, Aichele P. Effector T-cell differentiation during viral and bacterial infections: Role of direct IL-12 signals for cell fate decision of CD8+ T cells. Eur J Immunol. 2009 Jun 22;39(7):1774–83.

96. Starbeck-Miller GR, Xue H-H, Harty JT. IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo. Journal of Experimental Medicine. 2014 Jan 13;211(1):105–20.

97. Singh A, Mohan A, Dey AB, Mitra DK. Inhibiting the programmed death 1 pathway rescues Mycobacterium tuberculosis-specific interferon γ-producing T cells from apoptosis in patients with pulmonary tuberculosis. J INFECT DIS. 2013 Aug 15;208(4):603–15.

98. Fröhlich A, Marsland BJ, Sonderegger I, Kurrer M, Hodge MR, Harris NL, et al. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. Blood. 2007 Mar 1;109(5):2023–31.

99. Holst J, Szymczak-Workman AL, Vignali KM, Burton AR, Workman CJ, Vignali DAA. Generation of T-cell receptor retrogenic mice. Nat Protoc. 2006;1(1):406–17.

Chapter 4:

Discussion

Summary of findings

As an experimental tool, 1:1 mixed bone marrow chimeras are particularly useful for examining the effects of cytokine receptors on T cell subsets. The main advantage of this system is that it allows for the direct comparison of WT and KO cells within the same inflammatory environment. We used 1:1 mixed bone marrow chimeras to examine the effects of four key cytokines on $CD8^+$ T cell function during tuberculosis: IL-12, IL-21, IL-27, and type 1 IFN. Given that all of these cytokines are induced during tuberculosis, it is easy to imagine they have redundant roles during infection. We were surprised to learn that every cytokine supported $CD8^+$ T cell expansion, albeit to varying degrees. This suggests that $CD8^+$ T cells respond to the entire inflammatory milieu present in the lungs with each cytokine making some contribution to the overall response. The bacterial pathogen *Listeria monocytogenes* is similar in this regard, as $CD8^+$ T cells respond to both IL-12 and type 1 IFN during this infection (1). However, $CD8^+$ T cells do not require IL-21 to respond to *L. monocytogenes* (2), indicating the cytokine networks regulating $CD8^+$ T cells during tuberculosis are even more complex.

In addition to expansion, all of these cytokines were required for the efficient induction of granzyme B. This observation was consistent across every model used (1:1 chimeras, 4:1 chimers, and retrogenic cells), and led us to hypothesize that each signal was required for optimal cytolytic activity. *In vivo* CTL assays were performed and revealed that only the loss of IL-12 reduced the specific killing of target cells. Further analysis indicated that this reduction in killing was likely the result of diminished CTL numbers, not the cytotoxicity of the individual $CD8^+$ T cells. These findings caution

against the use of granzyme B as a surrogate for cytolytic function. Indeed, there are multiple pathways of cytolysis, and it is likely that a reduction in granzyme B alone fails to have a dramatic effect. These observations suggest that CTL activity is robust during tuberculosis and is likely supported by redundant cytokine signals. We have not examined the effect of IL-21 on cytolytic activity, but other data indicate IL-21 promotes perforin expression and cytolysis *in vitro* (Nunes-Alves C, unpublished observation) (3). Thus, it is possible that IL-21 will have a greater impact on cytolytic activity, but this remains to be tested.

Of the cytokines examined, type 1 IFN and IL-27 had the least dramatic effects overall. Both are dispensable in the lymph node, suggesting these cytokines do not support priming. It is possible they are not present in the lymph node during priming, and we are now conducting experiments to examine their expression at early time points after infection. In the lungs, type 1 IFN augments the expansion of $CD8^+$ T cells, but has no significant influence on $CD8^+$ T cell differentiation or IFN-γ production. IL-27 appears to have the smallest impact on expansion and was also dispensable for IFN-γ production. This latter finding was surprising, because IL-27 is required to promote IFN-γ expression in other infections (4). As for differentiation, IL-27 suppresses CD127 expression without promoting the accumulation of SLECs. This suggests it is dispensable for the terminal differentiation of effector cells during tuberculosis but may limit the accumulation of memory precursors. Overall, these observations are interesting, because both type 1 IFN and IL-27 suppress T_H1 responses *in vivo* (5-8). Thus, cytokines that dampen $CD4^+$ T cell responses can simultaneously expand $CD8^+$ T

cells. Balancing inflammation is essential during tuberculosis, and these cytokines may serve to fine tune inflammation in the lungs.

IL-12 and IL-21 had the greatest impact on $CD8^+$ T cell expansion in the lungs of 1:1 chimeras. Additionally, both cytokines are necessary to expand retrogenic $CD8^+$ T cells in the lymph node and act as signal 3 cytokines during tuberculosis. Given the general importance of IL-12, it is logical that it supports $CD8^+$ T cells, and these data indicated IL-12 is essential for the overall αβ T cell response to Mtb. However, both $CD8^+$ T cell priming and expansion occur in the absence of IL-12, indicating it is not absolutely required to generate a $CD8^+$ T cell response. It is possible that other signals can compensate for the loss of IL-12, and we plan to examine $CD8^+$ T cells deficient for both IL-12R and IFNAR.

The results with IL-21 are unexpected and, in some ways, contradict observations made in other infections. IL-21 is essential for $CD8^+$ T cell responses during chronic infections; however, it is not typically needed during priming (9-13). In our experiments, IL-21 is required after day 13 of infection, which is shortly after the initiation of $CD8^+$ T cell expansion in the lymph node. Thus, Mtb is unique among chronic pathogens in that $CD8^+$ T cells require IL-21 throughout the infection.

The requirements for IL-12 and IL-21 are somewhat different in the lymph node following low-dose aerosol infection. We observed that IL-$12R^{-/-}$ $CD8^+$ T cells underperform relative to WT cells at all time points and do not dilute their proliferation dye as quickly. This suggests IL-12 is immediately needed for efficient $CD8^+$ T cell priming, and IL-12 likely supports $CD8^+$ T cell expansion at all time points. Activated

DCs are the primary source of IL-12 in the lymph node, thus this cytokine should be present as soon as antigen is presented to naïve cells.

In contrast, naïve IL-21R$^{-/-}$ CD8^{+} T cells efficiently dilute their proliferation dye by day 11 and only start to lag behind WT cells by day 13. Because IL-21 is derived from CD4^{+} T cells rather than DCs, it is logical that T cell priming must first occur for IL-21 to accumulate in the lymph node. This may explain the delayed role for IL-21 and suggests CD4^{+} T cells directly support CD8^{+} T cell priming. Based on our data, it is unclear if IL-21 is supporting cell division or cell survival, and future experiment will need to address this issue. It is thought that signal 3 cytokines facilitate the continued expansion of cells directly following priming (14); however, IL-21 is also associated with preventing cell death in the early stages of vaccinia virus infection (15). In response to Mtb, it is possible IL-12 promotes cell division during priming while IL-21 maintains cell survival, but this hypothesis remains to be tested.

In addition to supporting priming, IL-12 and IL-21 also promote IFN-γ production. During tuberculosis, IFN-γ^{+} CD8^{+} T cells are predominantly KLRG1^{hi} in WT mice (Booty MG, unpublished observation), and we observe that IL-12 is essential for the differentiation of KLRG1^{hi} SLECs. This indicates that IL-12 drives both terminal differentiation and effector function during tuberculosis, and this link is frequently seen in other infections (1). In contrast, IL-21 differs from other signal 3 cytokines, because it has a mild effect on CD8^{+} T cell differentiation, yet is essential for expansion and cytokine production. In the absence of IL-21, the percentage of SLECs actually increases, suggesting this cytokine limits the accumulation of SLECs. Thus, IL-21

supports expansion and effector function without promoting the terminal differentiation of effector cells.

Beyond the initiation of the adaptive immune response, IL-21 is essential to prevent $CD8^+$ T cell exhaustion during tuberculosis. This observation was made in IL-$21R^{-/-}$ mice at 16 weeks post-infection. We observed that IL-$21R^{-/-}$ $CD8^+$ T cells had dramatically increased levels of PD-1 expression and fail to produce TNF at this time. The precise role of $CD8^+$ T cell exhaustion during tuberculosis in unclear, buy many speculate that it may limit bactericidal activity during chronic infection (16). Thus, it is exciting to identify a cytokine that prevents $CD8^+$ T cell exhaustion during Mtb infection. Though IL-$21R^{-/-}$ mice had unaltered numbers of $CD4^+$ T cells at week 4, $CD4^+$ T cells declined sharply by week 16. This indicates that IL-21 has multiple effects during chronic disease. Finally, IL-$21R^{-/-}$ mice eventually fail to control bacterial growth; indicating IL-21 is an important mediator of resistance to tuberculosis. The loss of bacterial growth is likely related to the reduced number of both $CD4^+$ and $CD8^+$ T cells, but this possibility will require further examination.

Implications and future directions

Infection with Mtb induces a considerable amount of inflammation, and in many ways, the $CD8^+$ T cell response reflects this. In general, increased levels of inflammation enhance the accumulation of effector $CD8^+$ T cells (17), and we observed that multiple inflammatory cytokines support $CD8^+$ T cell expansion during tuberculosis. However, this increase in the effector cell response can negatively impact the number of cells that will adopt a memory phenotype (17). This has been clearly demonstrated in *L. monocytogenes* infection. Pre-treatment of mice with antibiotics dampens inflammation during infection and results in a diminished effector $CD8^+$ T cell response (18). However, the $CD8^+$ T cells primed in this less inflammatory environment are prone to adopt a memory phenotype and respond more robustly to rechallenge. This experiment clearly illustrates how the generation of a potent effector response can be at odds with memory development.

Following Mtb infection, we observed that WT $CD8^+$ T cells overwhelmingly adopt a SLEC phenotype. Thus, it appears that high levels of inflammation, largely driven by IL-12, are promoting the terminal differentiation of $CD8^+$ T cells. This likely has profound effects on memory formation. There is little evidence suggesting people develop protective memory to tuberculosis. In fact, people who have been treated for tuberculosis are at a higher risk of developing disease again (19-23). These observations could be the result of recurrent disease, but it still raises the question of whether memory formation is undermined during the course of Mtb infection.

For the purposes of vaccine design, it is essential to consider the role of inflammation. Our research focused on the effects of IL-12, IL-21, IL-27, and type 1 IFN on primary $CD8^+$ T responses, but the data have implications for vaccine-elicited cells and potentially memory responses. Our results indicate that IL-12 is potent driver of $CD8^+$ T cell expansion and effector function during tuberculosis. Thus, adjuvant-induced IL-12 will generate a vigorous primary response to vaccination; however, it will also favor the production of terminally differentiated SLECs. IL-12 is undoubtedly essential for a robust $CD8^+$ T cell response, but too much will likely undermine the goal of generating long-lived memory lives.

IL-27 and type 1 IFN are also unlikely candidates to augment vaccine efficacy. During infection, they played minor roles in promoting expansion and appeared to have little effect on effector function. Furthermore, IL-27 actually inhibits CD127 expression, suggesting it may limit memory formation. Indeed, it would be interesting to determine of $IL\text{-}27R^{-/-}$ memory $CD8^+$ T cells respond better to rechallenge with Mtb. We have preliminary data in which we've antibiotic treated WT:$IL\text{-}27R^{-/-}$ 1:1 mixed bone marrow chimeras following infection. In these mice, a greater percentage of $IL\text{-}27R^{-/-}$ $CD8^+$ T cells adopt a central memory phenotype after 12 weeks of antibiotic treatment. Thus, the absence of IL-27 signaling may promote memory formation during tuberculosis.

Of all the cytokines tested, IL-21 appears to be the best candidate for enhancing vaccine efficacy. In primary infection, IL-21 promotes the expansion of $CD8^+$ T cells and their acquisition of effector functions. Most importantly, IL-21 does not promote the accumulation of SLECs, potentially making it an ideal cytokine to facilitate memory formation. If IL-21 can enhance the primary response to vaccination without terminally

differentiating $CD8^+$ T cells, it may generate a larger memory pool. In tumor vaccination models, IL-21 has already been demonstrated to enhance $CD8^+$ T cell function (24). Therefore, vaccine strategies that incorporate IL-21 should be examined in tuberculosis. Our experiments did not directly address vaccination, but we believe our data regarding the role of inflammation during disease may prove valuable in this regard.

We demonstrated a significant role for IL-21 in regulating $CD8^+$ T cells during tuberculosis as well as mediating resistance to disease. There are still many unanswered questions regarding the effects of IL-21 and research in this area will likely generate more exciting discoveries. First, the effects of IL-21 on CTL function should be fully addressed. Specifically, the effects of IL-21 on perforin expression should be examined during tuberculosis, and the cytolytic capacity of IL-$21R^{-/-}$ $CD8^+$ T cells needs to be assessed, either *in vivo* or *in vitro.*

The role of IL-21 in preventing exhaustion also requires intense examination. Following aerosol infection, C57BL/6 mice are able to control infection for over a year but inevitably succumb to disease. We still do not understand the events that lead to mortality in these mice, but many have questioned if the adaptive immune response fails late during infection. Could T cell exhaustion lead to unrestricted bacterial growth? $CD8^+$ T cell depletion only increases susceptibility after 200 days of infection, suggesting $CD8^+$ T cells mediate their protective effects later during disease. Therefore, IL-21 may be essential to maintain these protective effector functions at later time points. This is one of many possible interpretations of these observations, but it will be interesting to measure IL-21 levels in the lungs of infected mice over the extended

course of disease. Any significant decline in IL-21 concentrations in the lungs could trigger $CD8^+$ T cell exhaustion.

There are likely many mechanisms contributing to the increased susceptibility observed in IL-$21R^{-/-}$ mice. By week 16 of infection, both $CD4^+$ and $CD8^+$ T cells are reduced in the lungs, indicating a substantial decline in the overall T cell response. At present, the reasons for this decline in $CD4^+$ T cell numbers are unclear. IL-21 is not typically associated with maintaining $CD4^+$ T cells in other infections, but it does make an important contribution to the generation of T_H17 and Tfh cells. In the discussion section of chapter 3, we propose a role for IL-21 in promoting Tfh cell responses during chronic disease. IL-21 is not essential for the differentiation of Tfh cells, but in *T. gondii* infection, it does maintain Tfh cells during chronic disease (12). During tuberculosis, Tfh-like cells are associated with protection and, in particular, may serve to maintain organized lesions in the lungs (25). In future experiments, we will address the numbers of Tfh cells in the lungs of WT and IL-$21R^{-/-}$ mice throughout infection.

Across all infections examined, IL-21 is essential for the generation of plasma cells and the production of IgG (12,13,26-28). During tuberculosis, mice that cannot secrete antibodies ($AID^{-/-}$ $\mu S^{-/-}$) have a limited ability to control bacterial growth at later time points and succumb early to disease (29). Thus it is possible that absence of functional plasma cells is also contributing to susceptibility. All of these possibilities will require examination as we continue to explore the roles of IL-21 in immunity to Mtb.

Reference

1. Keppler SJ, Rosenits K, Koegl T, Vucikuja S, Aichele P. Signal 3 cytokines as modulators of primary immune responses during infections: the interplay of type I IFN and IL-12 in CD8 T cell responses. PLoS ONE. 2012;7(7):e40865.

2. Ertelt JM, Johanns TM, Rowe JH, Way SS. Interleukin (IL)-21-independent pathogen-specific CD8+ T-cell expansion, and IL-21-dependent suppression of CD4+ T-cell IL-17 production. Immunology. 2010 Oct;131(2):183–91.

3. Sutherland APR, Joller N, Michaud M, Liu SM, Kuchroo VK, Grusby MJ. IL-21 promotes CD8+ CTL activity via the transcription factor T-bet. The Journal of Immunology. 2013 Apr 15;190(8):3977–84.

4. Mayer KD, Mohrs K, Reiley W, Wittmer S, Kohlmeier JE, Pearl JE, et al. Cutting edge: T-bet and IL-27R are critical for in vivo IFN-gamma production by CD8 T cells during infection. J Immunol. 2008 Jan 15;180(2):693–7.

5. Hölscher C, Hölscher A, Rückerl D, Yoshimoto T, Yoshida H, Mak T, et al. The IL-27 receptor chain WSX-1 differentially regulates antibacterial immunity and survival during experimental tuberculosis. J Immunol. 2005 Mar 15;174(6):3534–44.

6. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, et al. Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta. Proc Natl Acad Sci USA. 2001 May 8;98(10):5752–7.

7. Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ, et al. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res. 2005 Nov;25(11):694–701.

8. Ordway D, Henao-Tamayo M, Harton M, Palanisamy G, Troudt J, Shanley C, et al. The hypervirulent Mycobacterium tuberculosis strain HN878 induces a potent TH1 response followed by rapid down-regulation. J Immunol. 2007 Jul 1;179(1):522–31.

9. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. Science. 2009 Jun 19;324(5934):1569–72.

10. Fröhlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. Science. 2009 Jun 19;324(5934):1576–80.

11. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral

infection. Science. 2009 Jun 19;324(5934):1572–6.

12. Stumhofer JS, Silver JS, Hunter CA. IL-21 is required for optimal antibody production and T cell responses during chronic Toxoplasma gondii infection. PLoS ONE. 2013;8(5):e62889.

13. Pérez-Mazliah D, Ng DHL, Freitas do Rosário AP, McLaughlin S, Mastelic-Gavillet B, Sodenkamp J, et al. Disruption of IL-21 Signaling Affects T Cell-B Cell Interactions and Abrogates Protective Humoral Immunity to Malaria. PLoS Pathog. 2015 Mar;11(3):e1004715.

14. Starbeck-Miller GR, Xue H-H, Harty JT. IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo. Journal of Experimental Medicine. 2014 Jan 13;211(1):105–20.

15. Novy P, Huang X, Leonard WJ, Yang Y. Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection. The Journal of Immunology. 2011 Mar 1;186(5):2729–38.

16. Behar SM, Carpenter SM, Booty MG, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. Seminars in Immunology. 2014 Dec;26(6):559–77.

17. Butler NS, Nolz JC, Harty JT. Immunologic considerations for generating memory CD8 T cells through vaccination. Cell Microbiol. 2011 Jul;13(7):925–33.

18. Badovinac VP, Harty JT. Manipulating the rate of memory CD8+ T cell generation after acute infection. J Immunol. 2007 Jul 1;179(1):53–63.

19. van Rie A, Warren R, Richardson M, Victor TC, Gie RP, Enarson DA, et al. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N Engl J Med. 1999 Oct 14;341(16):1174–9.

20. Dobler CC, Crawford ABH, Jelfs PJ, Gilbert GL, Marks GB. Recurrence of tuberculosis in a low-incidence setting. European Respiratory Journal. 2009 Jan;33(1):160–7.

21. Crofts JP, Andrews NJ, Barker RD, Delpech V, Abubakar I. Risk factors for recurrent tuberculosis in England and Wales, 1998-2005. Thorax. 2010 Apr;65(4):310–4.

22. Millet J-P, Orcau A, de Olalla PG, Casals M, Rius C, Caylà JA. Tuberculosis recurrence and its associated risk factors among successfully treated patients. J Epidemiol Community Health. 2009 Oct;63(10):799–804.

23. Millet J-P, Shaw E, Orcau A, Casals M, Miró JM, Caylà JA, et al. Tuberculosis recurrence after completion treatment in a European city: reinfection or relapse? PLoS ONE. 2013;8(6):e64898.

24. Ma H-L, Whitters MJ, Konz RF, Senices M, Young DA, Grusby MJ, et al. IL-21 activates both innate and adaptive immunity to generate potent antitumor responses that require perforin but are independent of IFN-gamma. J Immunol. 2003 Jul 15;171(2):608–15.

25. Slight SR, Rangel Moreno J, Gopal R, Lin Y, Fallert Junecko BA, Mehra S, et al. CXCR5 T helper cells mediate protective immunity against tuberculosis. J Clin Invest. 2013 Feb;123(2):712–26.

26. Ozaki K, Spolski R, Feng CG, Qi C-F, Cheng J, Sher A, et al. A critical role for IL-21 in regulating immunoglobulin production. Science. 2002 Nov 22;298(5598):1630–4.

27. King IL, Mohrs K, Mohrs M. A nonredundant role for IL-21 receptor signaling in plasma cell differentiation and protective type 2 immunity against gastrointestinal helminth infection. The Journal of Immunology. 2010 Nov 15;185(10):6138–45.

28. Rasheed MAU, Latner DR, Aubert RD, Gourley T, Spolski R, Davis CW, et al. Interleukin-21 is a critical cytokine for the generation of virus-specific long-lived plasma cells. Journal of Virology. 2013 Jul;87(13):7737–46.

29. Torrado E, Fountain JJ, Robinson RT, Martino CA, Pearl JE, Rangel Moreno J, et al. Differential and site specific impact of B cells in the protective immune response to Mycobacterium tuberculosis in the mouse. PLoS ONE. 2013;8(4):e61681.

Appendix 1:

Cyclooxygenase-2 is critical for resistance to *Mycobacterium tuberculosis* infection

This work is adapted in part from an unpublished manuscript pending submission. The work presented included assistance from the following individuals: Maziar Divangahi (McGill University, Montreal, Quebec, Canada) and Kristen N. Peters (University of Massachusetts Medical School, Worcester, MA, USA).

Abstract

Prostanoids are a diverse family of lipid signaling molecules generated by cyclooxygenases (COX-1 and COX-2) and are important mediators of inflammation. In particular, the prostanoid prostaglandin E2 (PGE_2) is associated with multiple protective effects during *Mycobacterium* tuberculosis infection; however, mice deficient in PGE_2 production are only mildly susceptible to disease. To more completely address the role of prostanoid production during tuberculosis, we examine the susceptibility of mice deficient in either COX-1 or COX-2. We determined that COX-2 is essential for resistance, while COX-1 is largely dispensable. Mice lacking COX-2 initially control bacterial growth in the lungs, but show signs of increased inflammation and bacterial burden during chronic disease. Through the use of bone marrow chimeras, we determine if COX-2 is needed in hematopoietic or nonhematopoietic cells. Surprisingly, COX-2 expression is required in both groups of cells for optimal host resistance. Mice lacking COX-2 in either hematopoietic or nonhematopoietic cells have increased bacterial burden concurrent with increased immunopathology. These data implicate COX-2 as an important mediator of resistance and highlight a vital role for nonhematopoietic cells in the host response to tuberculosis.

Introduction

Overview of eicosanoids

Eicosanoids are a diverse family of signaling molecules derived from arachidonic acid (AA) that encompasses prostaglandins, thromboxanes, leukotrienes, and lipoxins. Together, prostaglandins and thromboxanes are included in a group of molecules referred to as prostanoids. Among their many functions, these lipid mediators play vital roles in regulating both innate and adaptive immune responses. Eicosanoid production is rapidly induced in innate immune cells in response to a number of stimuli, including bacterial infection, and these signals exert their effects in both an autocrine and paracrine manner (1). Though the effect of each eicosanoid is context dependent, leukotrienes are largely pro-inflammatory while lipoxins are involved in resolving inflammation (2). It is more challenging to generalize the effects of prostaglandins, which vary considerably.

All eicosanoids are made from arachidonic acid, which is generated from membrane phospholipids by the action of phospholipase A2 enzymes (PLA2). Arachidonic acid is then further metabolized in one of three main biosynthetic pathways involving cyclooxygenases, lipoxygenases, or P-450 epoxygenase (1). Prostanoids are generated by cyclooxygenases, which exist in two forms: COX-1, which is constitutively expressed in most mammalian tissues; and COX-2, which is induced in response to a number of proinflammatory stimuli (3). Both cyclooxygenases catalyze the conversion of arachidonic acid into PGH_2, which is the metabolic precursor of all prostanoids. Lipoxygenases are a larger group of enzymes, whose coordinated action can generate

multiple molecules including lipoxin A4 (LXA_4), lipoxin B4 (LXB_4), and a variety of leukotrienes. The effects of eicosanoids are diverse and have profound implications on balancing inflammatory processes in a number of settings.

The opposing roles of PGE_2 and LXA_4 on macrophage cell death

During tuberculosis, eicosanoids impact bacterial virulence through several mechanisms and are particularly important regulators of cell death. Indeed, the cell death modality of the infected macrophage can have profound effects on bacterial control and the outcome of disease. In general, apoptosis is thought to favor the host, and this form of cell death is frequently associated with reduced bacterial growth (4,5). We demonstrated that uninfected macrophages can kill Mtb by engulfing apoptotic cells containing bacteria, suggesting a mechanism to link apoptosis and bacterial control (6). During necrotic death, there is a breakdown of the cell's plasma membrane, and this is thought to facilitate the spread of bacteria. Several groups have reported that virulent H37Rv induces necrosis in infected macrophages while avirulent strains, including H37Ra, induce apoptosis (7-10).

H37Rv and H37Ra also differ in the eicosanoid species they elicit in infected macrophages, and this difference is relevant to the modality of cell death. Macrophages infected with avirulent H37Ra primarily produce prostaglandin E2 (PGE_2); however, infection with virulent H37Rv predominantly induces lipoxin A4 (LXA_4) (7-9,11). LXA_4 directly inhibits PGE_2 production during H37Rv infection by limiting the expression of COX-2, the inducible enzyme required to produce all prostaglandins. The role of eicosanoids in cell death was determined by adding exogenous PGE_2 and LXA_4 to

infected macrophages. The addition of PGE_2 to H37Rv-infected cells inhibits necrosis, and in avirulent H37Ra infection, the addition of LXA_4 drives down PGE_2 production and promotes necrotic death (11).

These results were confirmed using knockout mice lacking 5-lipoxygenase (5-$LO^{-/-}$ or $Alox5^{-/-}$), which is necessary for LXA_4 production, as well as mice lacking prostaglandin E synthase ($PTGES^{-/-}$), which is necessary to produce PGE_2. When infected with H37Rv, macrophages from 5-$LO^{-/-}$ mice predominantly die by apoptosis and almost no bacterial growth is seen by day seven post-infection (12). Conversely, $PTGES^{-/-}$ macrophages predominantly die by necrosis.

The ability of H37Rv to induce necrosis may be linked to its ability to cause membrane damage, including plasma membrane damage. Plasma membrane lesions are repaired by a process of lysosome exocytosis that is dependent on synaptotagmin-7 (Syt-7), a $Ca2^{+}$ sensor located on lysosomes (13,14). Syt-7 facilitates the fusion of lysosomal membranes with the damaged plasma membrane and repair can be assessed by the accumulation of lysosomal markers, including LAMP-1 and Syt-7, on the cell surface. Upon infection with H37Ra, both LAMP-1 and Syt-7 translocate to the cell surface; however, evidence of membrane repair is not observed during H37Rv infection (12). These results suggest H37Rv is inducing membrane damage and inhibiting lysosome-mediated repair pathways.

The ability of eicosanoids to overcome this block in membrane repair was examined by administering exogenous PGE_2 to macrophages infected with H37Rv. PGE_2 increased the expression of Syt-7 and was able to enhance plasma membrane repair during H37Rv infection (12). Macrophages from $PTGES^{-/-}$ mice fail to initiate

membrane repair in response to H37Ra, indicating that PGE_2 is required for Syt-7-mediated membrane repair during mycobacterial infection. Membrane repair is also related to cell death, because knocking down Syt-7 causes cells to undergo necrosis during H37Ra infection. These data suggest that the inhibition of PGE_2-driven membrane repair contributes to necrosis during infection with H37Rv.

PGE_2 functions by signaling through four distinct G-protein coupled receptors, EP1-EP4. We have established a role for both EP2 and EP4 signaling in protecting against necrosis. EP2 signaling primarily activates protein kinase A (PKA), and EP4 primarily signals through phosphatidylinositol-3 kinase (PI3K) (15). Pharmacological inhibition of PI3K inhibits PGE_2-driven membrane repair, suggesting this process is mediated by EP4 signaling (12). EP2 signaling also appears to inhibit necrosis, as EP2 is required for PGE_2 to protect against mitochondrial damage during infection with Mtb (11). Thus, PGE_2 and LXA_4 establish an axis that regulates macrophage cell death during tuberculosis.

The opposing roles of PGE_2 and LXA_4 on host susceptibility

There is considerable in vivo evidence to support this dichotomy of PGE_2 and LXA_4; however, these molecules have broad effects on inflammation. Thus, their actions *in vivo* cannot be reduced to only influencing macrophage cell death. In particular, lipoxins are potent anti-inflammatory mediators during infection and lipoxin production enhances the virulence of several pathogens (16,17). Both *Toxoplasma gondii* and *Pseudomonas aeruginosa* secrete enzymes with lipoxygenase activity and can directly generate lipoxins from host-derived substrates (18,19). These observations suggest that

manipulating lipoxin levels may be an important virulence strategy for multiple pathogens.

LXA_4 can be detrimental to adaptive immune response during tuberculosis through several mechanisms. By influence the cell death modality of infected cells, it delays cross presentation and, subsequently delays $CD8^+$ T cell priming (20). Additionally, LXA_4 directly inhibits IL-12 production by dendritic cells (DCs) (21). 5-$LO^{-/-}$ mice are more resistant to aerosol infection and have a decreased bacterial burden in the lungs (22). This resistance is associated with an enhanced T_H1 response resulting from increased IL-12 levels. Through these mechanisms, Mtb-elicited LXA_4 directly limits the host's ability to mount a protective adaptive immune response.

Studies using a zebrafish model of *Mycobacterium marinum* infection have identified additional consequences of increased LXA_4 signaling. Silencing an enzyme involved in leukotriene metabolism, leukotriene-A4 hyrdolase (LTA4H), results in the excess accumulation of LXA_4 and renders zebrafish larvae more susceptible to *M. marinum* infection (23). Further analysis, revealed that LTA4H is an important mediator of inflammation during mycobacterial infection through its ability to influence TNF levels. Reductions in LTA4H activity result in increased LXA_4 that directly limits TNF production and increases susceptibility. Conversely, heightened LTA4H activity results in increased levels of leukotriene B4 (LTB_4), which promotes TNF production and immunopathology (17,23,24). Thus, LTA4H regulates a delicate balance of inflammation that must be maintained for optimal host resistance.

The negative effects of lipoxin and leukotriene production are also supported in human studies. Variants in the gene encoding 5-LO (*ALOX5*) are associated with

increased susceptibility to active pulmonary tuberculosis (25). Though this study did not directly address 5-LO functionality in patients with tuberculosis, several of the variants were hypothesized to increase 5-LO expression. Polymorphisms in LTA4H are also associated with susceptibility to mycobacterial disease. In particular, heterozygosity of certain LTA4H single nucleotide polymorphisms (SNPs) is associated with protection from extra-pulmonary tuberculosis and severe forms of leprosy (23). Heterozygous individuals are hypothesized to produce intermediate amount of LTB_4, which balances inflammatory responses. This intermediate level of inflammation is thought to optimize bacterial control without generating immunopathology. The relevance of these finding in pulmonary tuberculosis is questionable (26,27), but this work underscores the importance of lipid mediators during human mycobacterial diseases.

Precisely defining the protective effects of PGE_2 *in vivo* during tuberculosis has proven challenging. We observed that $PTGES^{-/-}$ mice succumb more quickly to low dose aerosol infection and have a higher bacterial burden in the lungs at 5 weeks post-infection (*Figure A-1*). However, this increase in bacterial burden was transient and was not observed at later time points. Additionally, the susceptibility phenotype of $PTGES^{-/-}$ mice takes over 200 days to manifest itself, suggesting PGE_2 is not strictly required during the early stages of infection. Of course, knocking out PTGES has broader effects than simply eliminating PGE_2 levels. Research indicates that blocking PTGES activity does not reduce overall prostanoid production, but rather shifts the particular prostanoids generated in response to stimulation (28). $PTGES^{-/-}$ macrophages fail to produce PGE_2 in response to LPS stimulation but instead respond with increased levels

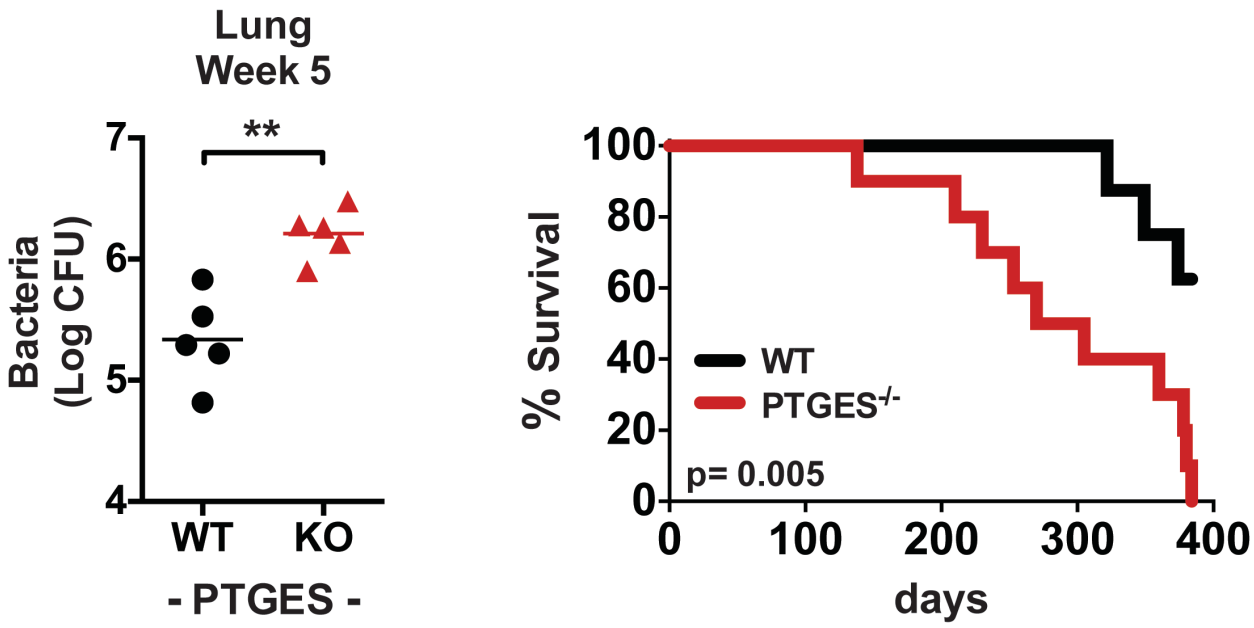

Figure A-1. PTGES$^{-/-}$ mice are less resistant to tuberculosis.

(Left) Bacterial burden in the lungs of WT and PTGES$^{-/-}$ mice 5 week after low dose aerosol infection. (n=5 mice per group) This increase in bacterial burden was transient and not observed at later time point during infection. (Right) Survival of WT and PTGES$^{-/-}$ mice following low dose aerosol infection (n=8-10 mice per group). Each point represents a single mouse and lines represent the mean. Data represent a single experiment.

of PGD_2 production. Thus, it possible that overproduction of other prostanoids confounds the interpretation of experiments involving $PTGES^{-/-}$ mice.

Recently, the protective effects of PGE_2 were demonstrated *in vivo* during tuberculosis. Interleukin-1 alpha and beta (IL-1α/β) are necessary for host resistance during tuberculosis, and both IL-$1\alpha^{-/-}$ and IL-$1\beta^{-/-}$ mice succumb early to disease (29,30). It is now apparent that PGE_2 is a major mediator of the protective effects of IL-1 (31). IL-1 stimulation of infected macrophages directly promotes PGE_2 production, and intranasal treatment with PGE_2 increases the control of bacterial growth in highly susceptible IL-$1\alpha^{-/-}$,IL-$1\beta^{-/-}$ (double knockout) mice. PGE_2 promotes bacterial control and protection by directly suppressing detrimental type 1 IFN production during disease (31). Overall, PGE_2 has a positive influence on host resistance and appears to act through a number of mechanisms *in vivo*.

Rationale for current studies

Given the protective roles of PGE_2 both *in vitro* and *in vivo*, it is puzzling that $PTGES^{-/-}$ mice are only mildly susceptible to tuberculosis. We speculate that the accumulation of other prostanoids in $PTGES^{-/-}$ mice is masking a susceptibility phenotype and seek to address the role of prostanoids in host resistance. We hypothesize that broadly limiting prostanoid production will increase susceptibility to tuberculosis. To this end, we examine the susceptibility of mice deficient in either COX-1 or COX-2, the upstream enzymes required for prostanoid production.

Initial studies indicated that COX-1 is dispensable for host resistance, but the absence of COX-2 dramatically increases susceptibility. Despite succumbing to

disease, COX-2$^{-/-}$ mice are capable of controlling bacterial growth at early time points and appear to mount a robust adaptive immune response. Thus, it appears unlikely that defects in the initial host immune response are contributing to susceptibility. COX-2$^{-/-}$ mice do have higher bacterial burdens at later time points, and this appears to directly precede their demise. To determine the requirements of COX-2 expression in hematopoietic and nonhematopoietic cell lineages, we generated a series of bone marrow chimeras and assessed their susceptibility. Surprisingly, optimal host resistance requires COX-2 expression in both hematopoietic and nonhematopoietic cells. Mice lacking COX-2 in a single compartment survive longer than mice lacking COX-2 in all cells; however, they have higher bacterial burden and increased immunopathology.

These data indicate that COX-2 expression is required in a variety of cell types to mediate resistance to tuberculosis. Additionally, COX-2 is unnecessary to control early bacterial growth and is dispensable for the initial immune response to Mtb. We hypothesize that COX-2 is necessary to maintain the proper balance of inflammation during chronic disease and promotes host survival by limiting immunopathology.

Results

Prelude: lessons from B6;129 Cox-2$^{-/-}$ mice

To begin understanding the full impact of prostanoids during Mtb infection, we examined the susceptibility of mice deficient in either cyclooxygenase 1 or 2 (COX-1$^{-/-}$ or COX-2$^{-/-}$). Currently, the only commercially available COX-1$^{-/-}$ and COX-2$^{-/-}$ mice are on a mixed genetic background (B6;129). This is because transferring the COX-2 null allele to a pure C57BL/6 background greatly reduces the viability these mice (32). Additionally, COX-2$^{-/-}$ females are infertile; so all breeding must be done with heterozygous animals. For this reason, all survival experiments utilized B6;129 littermates as controls. Furthermore, mice doubly deficient for COX-1 and COX-2 die shortly after birth, so the loss of both enzymes cannot be directly examined.

To assess susceptibility, COX-1$^{-/-}$, COX-2$^{-/-}$, and WT (B6;129) mice were infected with 90 CFU of virulent Mtb (Erdman strain) via the aerosol route and survival was monitored (*Figure A-2*). COX-2$^{-/-}$ mice exhibited a significant decrease in survival (median survival = 28 days) compared to WT and COX-1$^{-/-}$ mice (median survival >125 days). Encouraged by these findings, we set out to characterize the heightened susceptibility of COX-2$^{-/-}$ mice. Due to a technical problem, the mice in the follow-up experiment received a very low inoculum (approximately 5 CFU); however, this was fortuitous as it allowed us to examine COX-2$^{-/-}$ mice at much later time points. At this lower infectious dose, both COX-2$^{-/-}$ and WT mice survived and presented no overt signs of illness (data not shown). In place of monitoring survival, we examined bacterial burden and immune function in these mice at days 35 and 90. In the lungs, the data

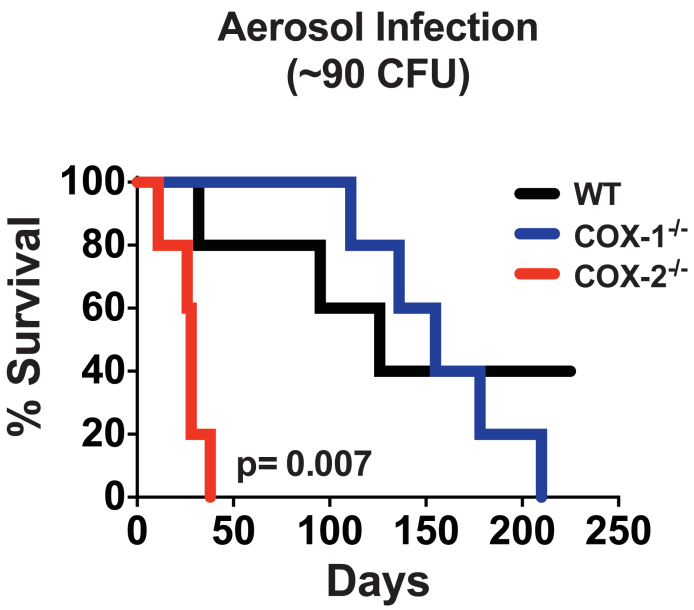

Figure A-2. COX-2, but not COX-1, is necessary for resistance to tuberculosis.

Survival of WT, COX-$1^{-/-}$, and COX-$2^{-/-}$ mice following aerosol infection with 90 CFU of Mtb (Erdman strain). All mice are on the B6;129 mixed genetic background. (n=5 mice per group) Data represent a single experiment.

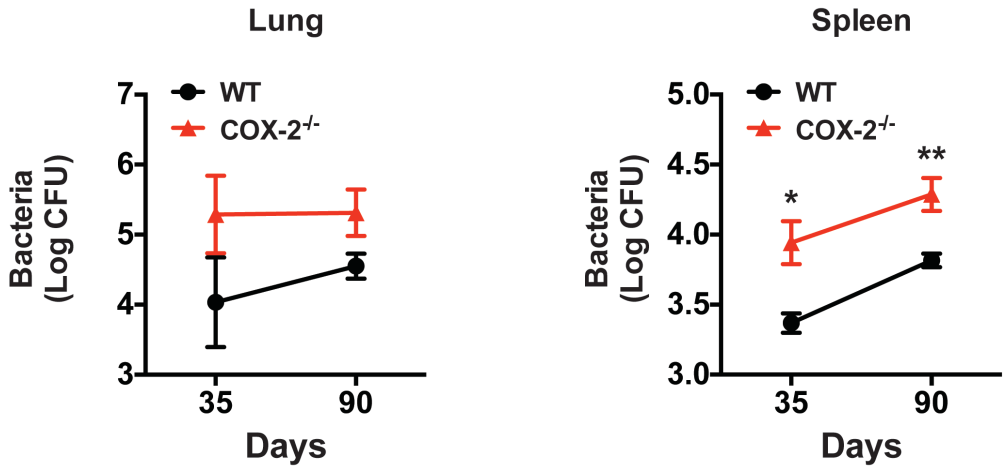

Figure A-3. At a lower inoculum, COX-$2^{-/-}$ mice survive following aerosol infection, but fail to control bacterial growth.

Bacterial burden in the lungs and spleens of WT and COX-$2^{-/-}$ mice (B6;129 background) following an extremely low dose aerosol infection (5 CFU). Each point represents the mean ± SEM (n=5 mice per group) *P < 0.05 and **P < 0.01 (unpaired Student's t-test). Data represent a single experiment.

were too variable to be conclusive, but the bacterial burden did appear higher in COX-2$^{-/-}$ mice (*Figure A-3*). In the spleens, COX-2$^{-/-}$ mice failed to control bacterial growth at both time points and burden was significantly increased. Other than bacterial burden, we identified no other differences between WT and COX-2$^{-/-}$ mice. COX-2$^{-/-}$ mice had comparable numbers of B, CD4^{+} T, and CD8^{+} T cells at both time points (data not shown). These data are the first to suggest COX-2 is required to control bacterial growth during tuberculosis, but provide few clues regarding the mechanism of this control.

Because COX-2 can be induced in nearly every cell type, we next determined if the enhanced susceptibility of COX-2$^{-/-}$ mice results from a loss of prostanoid production by hematopoietic cells. Bone marrow chimeras were made by transferring either WT or COX-2$^{-/-}$ bone marrow into lethally irradiated B6;129 F2 recipients. To assess susceptibility, chimeras were infected via intravenous injection, as this model of infection typically shortens the amount of time required to observe a survival phenotype. Surprisingly, COX-2$^{-/-}$ chimeras were actually more resistant to both low and high intravenous doses of virulent Mtb (*Figure A-4*). Bacterial burden was assessed in bone marrow chimeras three weeks after IV infection with a low inoculum (Day 1 = 70,000 CFU/spleen). At this time point, COX-2$^{-/-}$ bone marrow chimeras had significantly reduced bacterial burdens in the lungs and spleen (*Figure A-5*). It is likely that IV infection induces greater inflammation, and these data lead us to hypothesize that the loss of hematopoietic COX-2 reduced inflammation and prolonged survival during IV infection. In support of this, lung histology showed fewer infiltrating polymorphonuclear cells in the COX-2$^{-/-}$ chimeras (*Figure A-6A*), and flow cytometry confirmed the presence of fewer neutrophils in the lungs when hematopoietc COX-2 was absent (*Figure A-6B*).

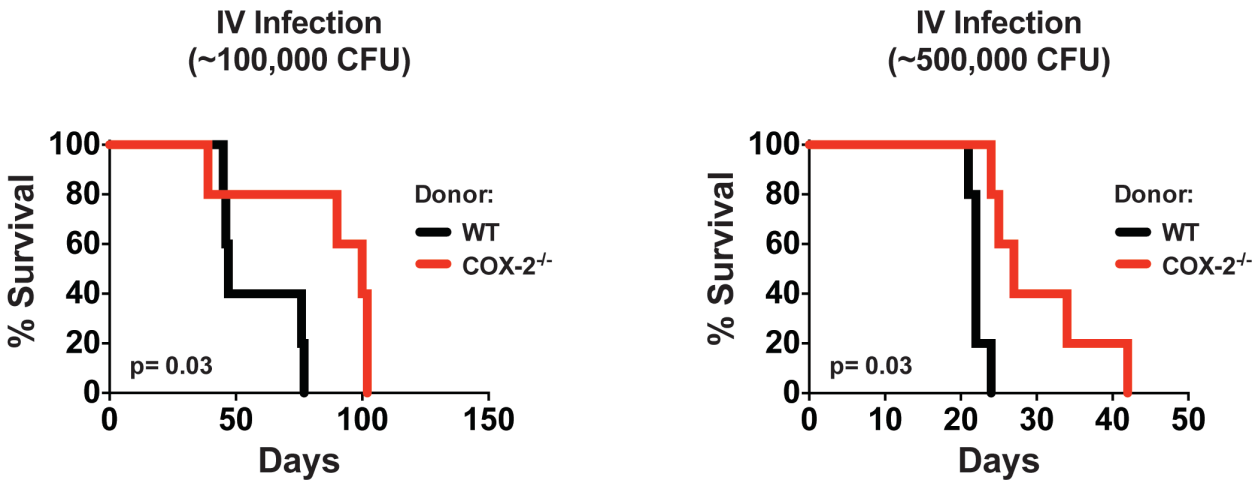

Figure A-4. Mice lacking hematopoietic COX-2 are more resistant to IV infection.

Survival of WT and COX-2$^{-/-}$ bone marrow chimeras following IV infection with the indicated inocula of Mtb (Erdman strain). All recipient mice are WT B6;129 (n=5 mice per group). Data are representative of two independent experiments. For IV infections, day 1 CFU was assessed in the spleen.

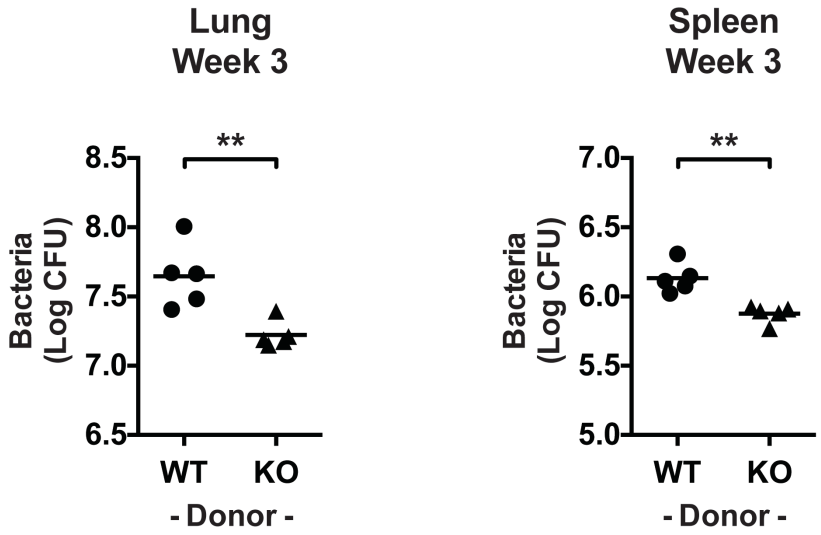

Figure A-5. Following IV infection, mice lacking hematopoietic COX-2 have reduced bacterial burdens.

Bacterial burden in the lungs and spleens of WT and COX-2$^{-/-}$ bone marrow chimeras (B6;129 background) following IV infection (Day 1 = 70,000 CFU/Spleen) Each point represents a single mouse and lines represent the mean (n=5 mice per group) *P < 0.05 and **P < 0.01 (unpaired Student's t-test). Data represent a single experiment.

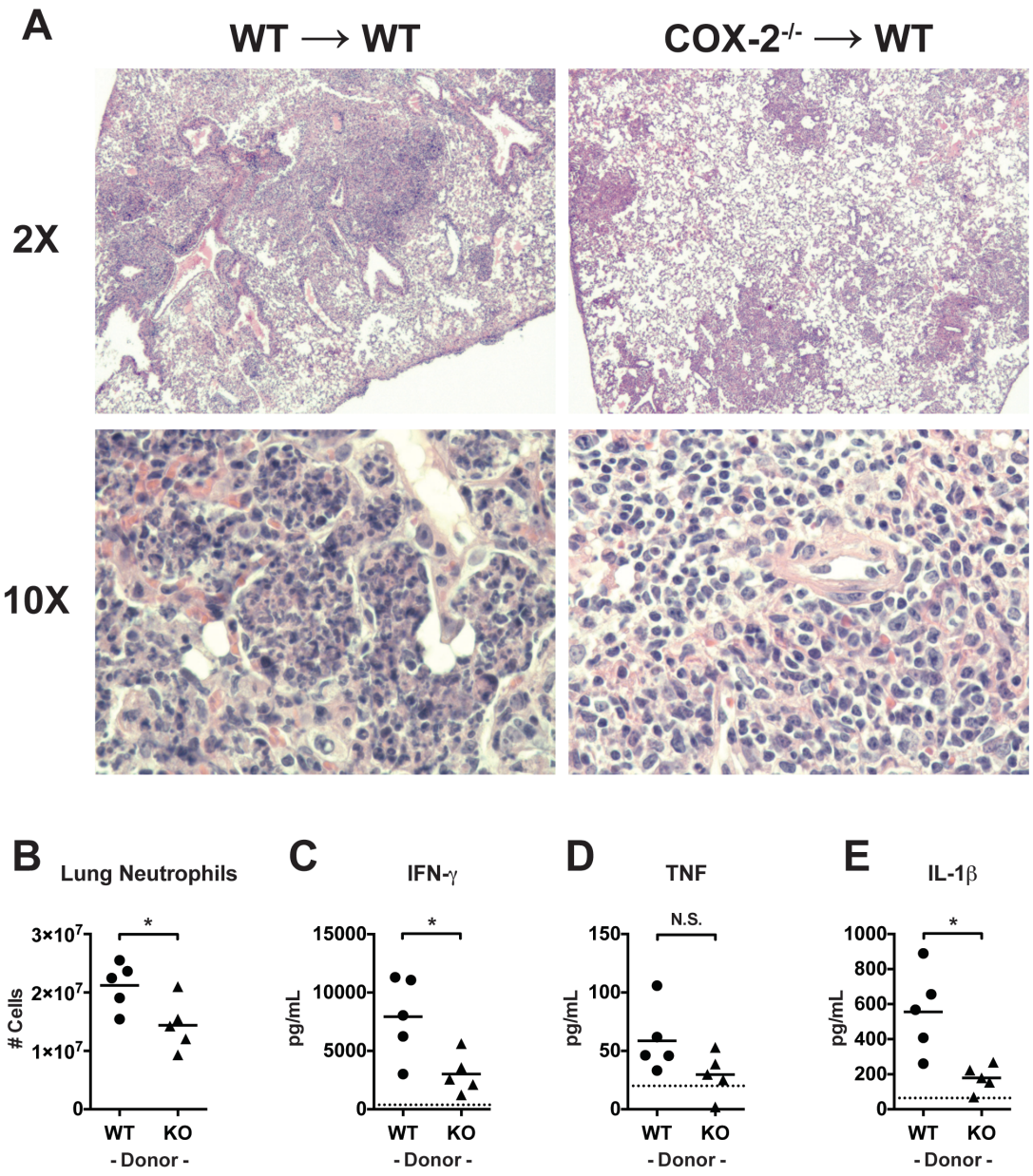

Figure A-6. Mice lacking hematopoietic COX-2 have reduced inflammation in the lungs following IV infection.

All data are from the indicated bone marrow chimeras 3 weeks after IV infection (Day 1 = 70,000 CFU/Spleen). (A) Representative H&E staining in the lungs of the indicated bone marrow chimeras. (B) Total number of neutrophils in the lungs (neutrophils are defined as $F4/80^{-}$ $CD11b^{+}$ $Gr1^{hi}$ cells). Total amounts of IFN-γ (C), TNF (D) and IL-1β (E) in the lung cell-free homogenates as determined by ELISA. Each point represents a single mouse and lines represent the mean (n=5 mice per group) *$P < 0.05$ (unpaired Student's t-test). Dashed lines represent the limit of detection for the ELISAs. Data represent a single experiment.

Furthermore, cell-free lung homogenate from COX-2$^{-/-}$ chimeras had significantly reduced levels of IFN-γ and IL-1β (*Figure A-6C and A-6E*). Collectively, these data suggest COX-2$^{-/-}$ chimeras incur less inflammation during IV infection, thus a decrease in immunopathology likely contributes to their survival advantage in this model.

The mixed genetic background (B6;129) of the COX-2$^{-/-}$ mice presents a unique challenge when working with these animals, as it is well established that mouse strains differ in their relative susceptibility to Mtb (33). The C57BL/6J (B6) strain is resistant to Mtb infection, while the 129 strain is generally considered more susceptible. Thus, the susceptibility of the B6;129 F2 mice was likely variable in our experiments. Indeed, variability was often high with these mice, leading us to conclude it was not a viable model to continue studying the role of COX-2 during infection. Still, these experiments generated several important observations. First, COX-2, but not COX-1, appears to be essential for resistance to tuberculosis. Second, studies with the IV infection model highlight the importance of hematopoietic COX-2 during inflammation. COX-2 is required for resistance, but it can also augment damaging inflammation under certain settings (34). Finally, these studies illustrate that IV and aerosol infections can have different outcomes, and suggest using caution when comparing these two models.

COX-2 is required for host resistance to tuberculosis

Fortunately, we were able to obtain COX-2$^{-/-}$ mice backcrossed 10 generations to a BALB/c background (35). BALB/c mice are resistant to tuberculosis, thus these mice are more suitable to assess COX-2 function. Following aerosol infection, BALB/c COX-2$^{-/-}$ mice are more susceptible that WT controls and have a median survival of ~57 days

(*Figure A-7*). This survival phonotype is less dramatic than that of B6;129 mice, but nonetheless implicates COX-2 is an important mediator of host resistance. Previous studies noted that mice heterozygous for the COX-2 null allele produce intermediate amounts of PGE_2 in response to stimulation (36). To test for a possible gene dosage effect, the survival of heterozygous mice (COX-$2^{+/-}$) was monitored, but no phenotype was noted (data not shown). Given that COX-$2^{-/-}$ is an immediate early gene, we reasoned that it plays an important role in the initial innate immune response to Mtb. Thus, we hypothesized that unrestricted bacterial growth was leading to the early demise of the COX-$2^{-/-}$ mice. Four weeks after infection, bacterial burden was comparable in WT mice and groups of mice homozygous and heterozygous for the COX-2 null allele (*Figure A-8*). These data indicate that COX-$2^{-/-}$ mice control bacterial growth at early time points, despite being less resistant to disease.

With the B6;129 mice, we serendipitously noted that COX-$2^{-/-}$ mice survive infection with a very low inoculum, but fail to control bacterial growth at later time points. In a similar experiment, we infected WT and COX-$2^{-/-}$ BALB/c mice with approximately 5 CFU of Mtb (Erdman strain). At this low dose, COX-$2^{-/-}$ BALB/c mice survived for at least 12 weeks and exhibited no overt signs of illness. At week 12, COX-$2^{-/-}$ mice had significantly higher amounts of bacteria in their lungs and spleens (*Figure A-9*). Though COX-$2^{-/-}$ mice control early bacterial replication, it appears that they fail to restrict bacterial growth at later time points during chronic disease.

In an effort to confirm this finding, COX-$2^{-/-}$ mice were infected with the standard dose of Mtb (100-200 CFU), and survival was closely monitored. We reasoned that increased bacterial growth directly precedes the demise of the mice, and waited until

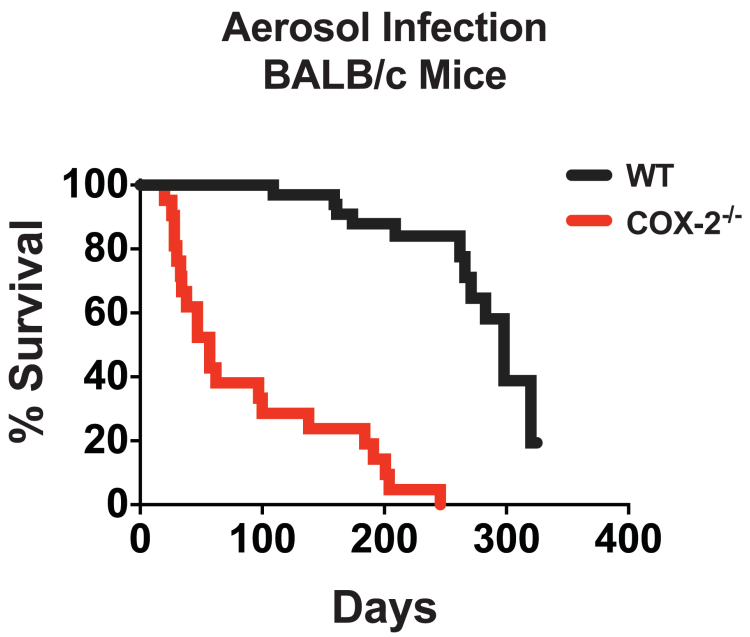

Figure A-7. COX-2 is required for resistance to tuberculosis.

Survival of WT and COX-$2^{-/-}$ BALB/c mice following low dose aerosol infection with Mtb (Erdman strain). Data are combined from four independent experiments. Median survival is 57 days for COX-$2^{-/-}$ mice and 298 days for WT mice ($p < 0.0001$). n= 21-33 mice per group.

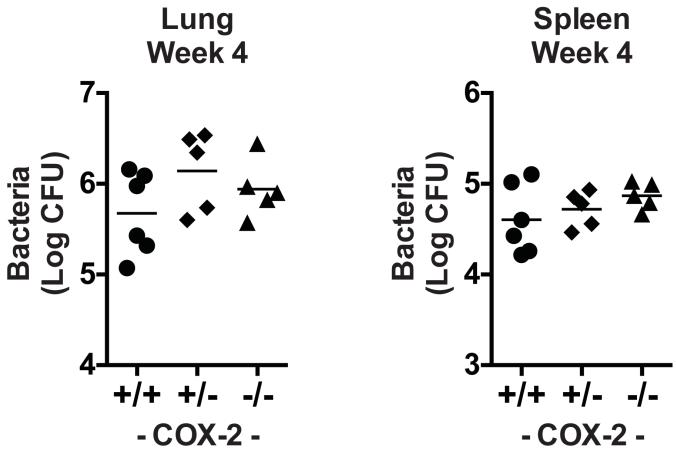

Figure A-8. COX-2 is not required to control bacterial growth at early time points.

Bacterial burden in the lungs and spleens of WT, COX-$2^{+/-}$, and COX-$2^{-/-}$ mice (BALB/c background) following low dose aerosol infection. Each point represents a single mouse and lines represent the mean (n=5-6 mice per group). Data are representative of two independent experiments.

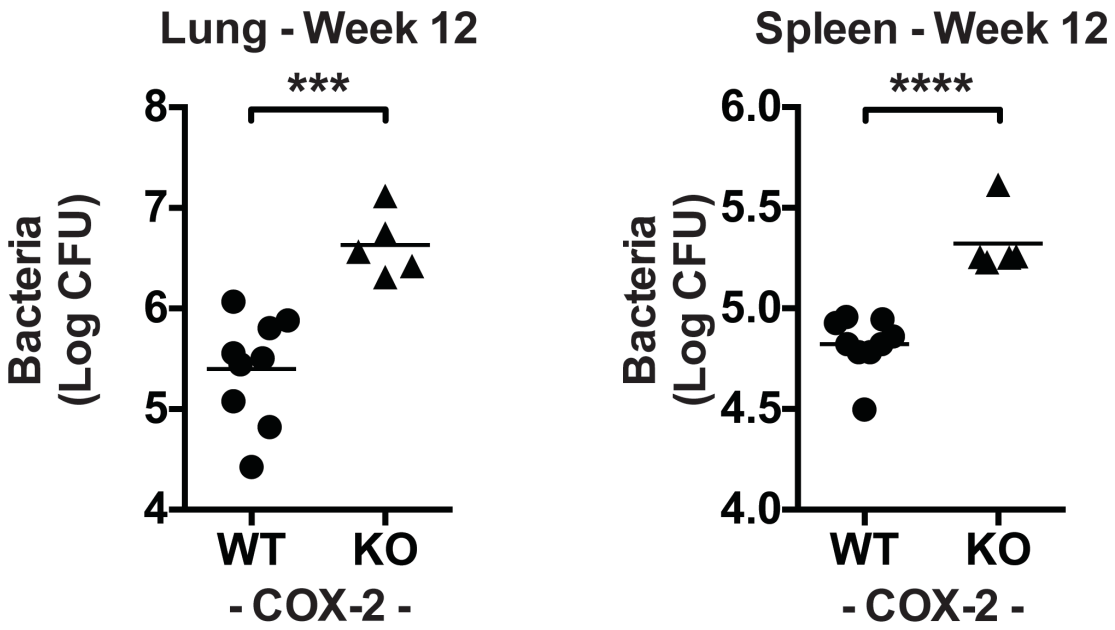

Figure A-9. At a lower inoculum, COX-2$^{-/-}$ mice survive following aerosol infection, but fail to control bacterial growth.

Bacterial burden in the lungs and spleens of WT and COX-2$^{-/-}$ mice (BALB/c background) following extremely low dose aerosol infection (5 CFU). Each point represents a single mouse and lines represent the mean (n=5-9 mice per group). ***P < 0.001 and ****P < 0.0001 (unpaired Student's t-test). Data represent a single experiment.

the COX-2$^{-/-}$ group started to succumb to disease. (*Figure A-10*). At week 9, the mice were sacrificed and bacterial burden was assessed. The data indicate that a subset of COX-2$^{-/-}$ mice had increased bacterial burden in the lungs, though this was not uniform throughout the COX-2$^{-/-}$ group (*Figure A-10*). We interpret these data to indicate bacterial burden precedes host demise in the COX-2$^{-/-}$ mice. Because COX-2$^{-/-}$ mice succumb to disease at different rates, it is challenging to capture a window of time in which the entire group of COX-2$^{-/-}$ mice have increased bacterial burden. It should also be noted that the immune response in COX-2$^{-/-}$ mice was monitored at every time point. No difference in the numbers of neutrophils, B cells, CD4^{+} T cells, or CD8^{+} T cells was ever noted (data not shown).

COX-2 expression is required in both hematopoietic and nonhematopoietic cells to mediate resistance to tuberculosis.

Because we observed no overt immunological phenotype in infected COX-2$^{-/-}$ mice, we hypothesized that COX-2 expression in nonhematopoietic cells may be involved in host resistance. To identify the COX-2-expressing cells mediating resistance, bone marrow chimeras were generated using BALB/c COX-2$^{-/-}$ mice. As an initial assessment, WT and COX-2$^{-/-}$ bone marrow was transferred into lethally irradiated WT recipients. Four weeks after infection, these two groups of bone marrow chimeras had comparable bacterial burden in the lungs (data not shown). This finding is unsurprising given that intact COX-2$^{-/-}$ mice are also comparable to WT controls at week four.

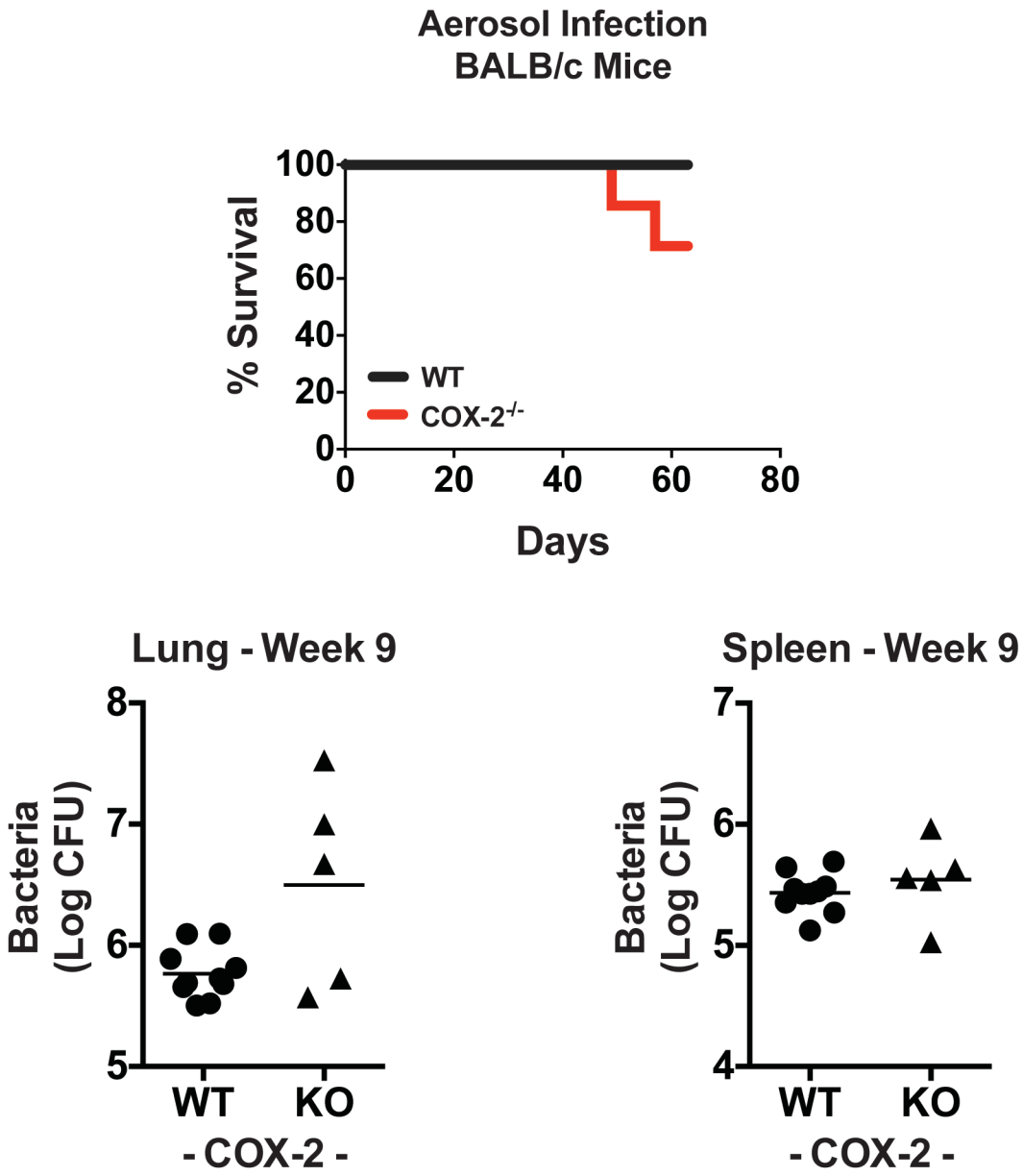

Figure A-10. Moribund COX-2$^{-/-}$ mice fail to control bacterial growth.

(Top) Survival analysis of the mice used for this experiment. Mice were sacrificed at week 9 to assess bacterial burden. This time point was chosen, because animals were beginning to succumb to disease. Bacterial burden in the lungs and spleens of WT and COX-2$^{-/-}$ mice (BALB/c background) at week 9 following low dose aerosol infection. Each point represents a single mouse and lines represent the mean (n=5-10 mice per group). Data represent a single experiment.

We next generated multiple combinations of bone marrow chimeras: WT bone marrow into WT recipients; WT bone marrow into COX-2$^{-/-}$ recipients; COX-2$^{-/-}$ bone marrow into WT recipients; and COX-2$^{-/-}$ bone marrow into COX-2$^{-/-}$ recipients. These mice were infected via the aerosol route and monitored for survival. COX-2$^{-/-}$ mice reconstituted with COX-2$^{-/-}$ bone marrow were the first to succumb and had a median survival comparable to intact COX-2$^{-/-}$ mice (*Figure A-11*). This observation confirmed that the process of generating bone marrow chimeras does not dramatically alter the susceptibility of these mice. It also indicates that COX-2 expression is required in both hematopoietic and nonhematopoietic cells for optimal resistance to tuberculosis. Over the course of the experiments, WT recipient mice survived regardless of the donor bone marrow received (*Figure A-11*). Intriguingly, COX-2$^{-/-}$ mice reconstituted with WT bone marrow had significantly decreased survival, suggesting non-hematopoietic COX-2 expression is important for resistance (*Figure A-11*).

At week 15, the surviving bone marrow chimeras were sacrificed and lung pathology was assessed. In WT into WT chimeras, lung pathology was minimal and consisted mainly of small diffuse myeloid lesions. In both KO into WT and WT into KO chimeras, a significantly larger area of lung tissue was occupied with large diffuse myeloid lesions and there were notable areas of necrosis (*Figure A-12*). Thus, mice lacking COX-2 in either hematopoietic or nonhematopoietic cells had significantly worse lung pathology. In addition to increased lung pathology, these two groups of chimeras had increased bacterial burdens in the lungs at week 15 (*Figure A-12*). Ultimately, these data show that both hematopoietic and nonhematopoietic cells have a role in mediating resistance to tuberculosis.

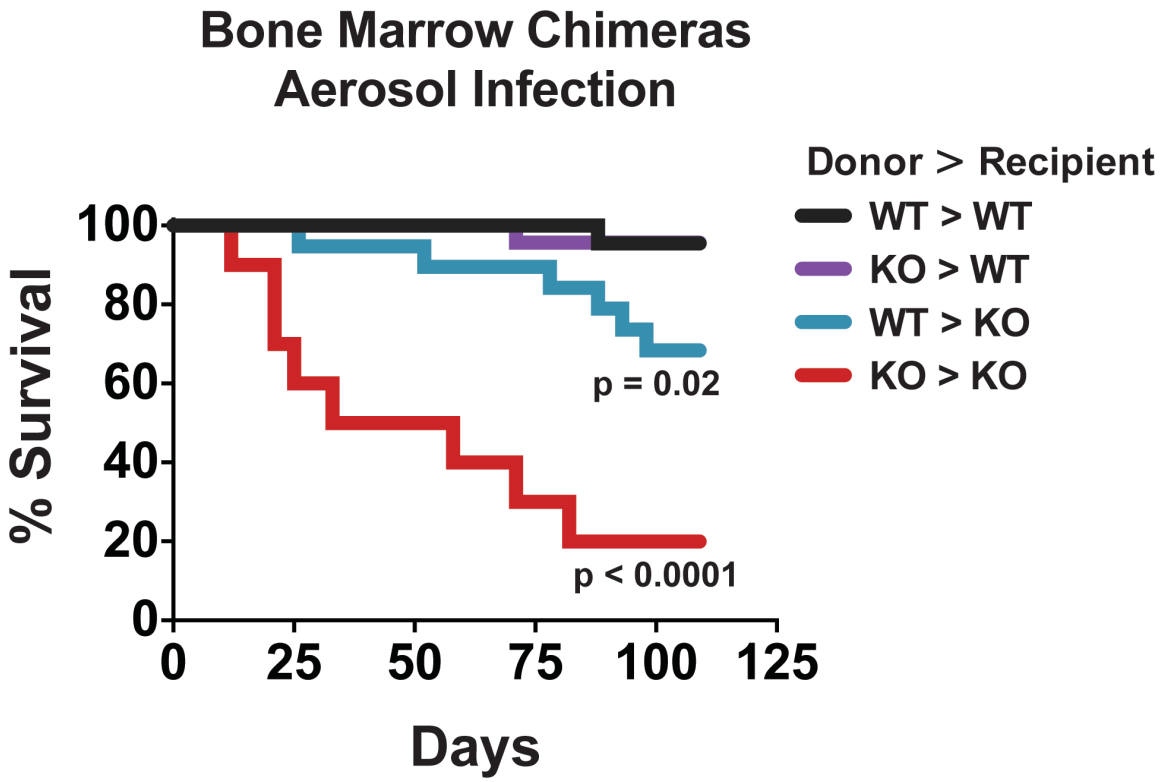

Figure A-11. Nonhematopoietic COX-2 mediates resistance to tuberculosis.

Survival of the indicated bone marrow chimeras following low dose aerosol infection with Mtb (Erdman strain). Data are combined from three independent experiments. Each experiment was terminated by day 110. n= 10-20 mice per group.

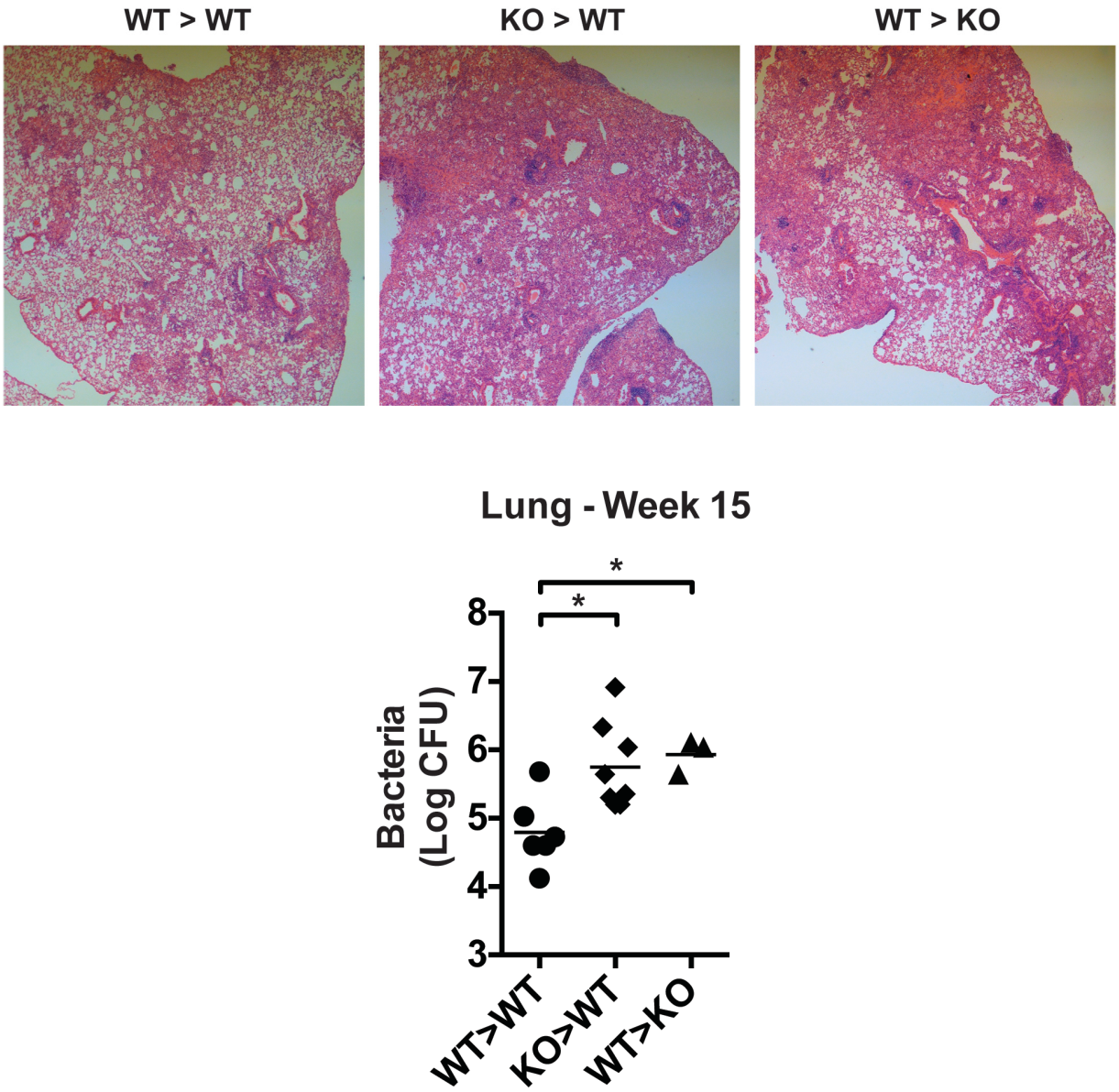

Figure A-12. COX-2 expression is required in both hematopoietic and non-hematopoietic cells to control bacterial growth and limit immunopathology.

All data are from the indicated bone marrow chimeras 15 weeks after low dose aerosol infection (Top) Representative H&E staining in the lungs of the indicated bone marrow chimeras at 2X magnification. (Bottom) Bacterial burden in the lungs of the indicated bone marrow chimeras at week 15. Each point represents a single mouse and lines represent the mean (n=3-7 mice per group) *P < 0.05 (one way ANOVA followed by Dunnett's multiple comparisons test). Data are representative of three independent experiments.

Discussion

PGE_2 is protective during tuberculosis through a variety of mechanisms, yet mice incapable of generating this molecule are only mildly susceptible to disease. We hypothesized that other prostanoids contribute to resistance, and examined mice deficient in COX-1 and COX-2. These two enzymes are comparable in their ability to convert arachidonic acid to PGH_2, the precursor of all prostanoids; however, they differ considerably in terms of their regulation. COX-1 is constitutively expressed in most cells and is generally associated with maintaining homeostatic prostanoid production (3). Conversely, COX-2 is rapidly induced following many forms of stress or activation, including infection. Based on our initial studies with B6;129 mice, COX-1 appears dispensable during tuberculosis, but COX-2 is essential to host resistance. This finding has since been replicated in several models. We confirmed the necessity of COX-2 using knockout mice backcrossed to the resistant BALB/c background, and there is also recently published data that supports these findings. Mice carrying a mutation that partially inactivates the enzymatic activity of COX-2 also succumb early to aerosol infection (31). Combined, these data provide compelling evidence for the importance of COX-2 during tuberculosis.

Initially, we hypothesized that the loss of COX-2 would impair the innate immune response, increase macrophage necrosis, and lead to unrestricted bacterial growth. However, COX-$2^{-/-}$ mice are able to control bacterial growth at early time points, suggesting the initial response to the invading bacteria is uncompromised. Thus, COX-2 is likely mediating susceptibility through mechanisms unrelated to the direct control of bacteria growth in macrophages.

When infected with a very low inoculum (~5 CFU), both B6;129 and BALB/c COX-2$^{-/-}$ mice survive much longer, likely because the progression of disease is slowed. These infections allowed us to examine bacterial burden at much later time points than normally possible, and indicated that COX-2$^{-/-}$ mice eventually fail to control bacterial growth. This leads us to speculate that bacterial burden only increases shortly before the mice succumb to disease. Indeed, mice infected with the standard inoculum (100-200 CFU) show signs of increased burden by week 9 post-infection, a time point when COX-2$^{-/-}$ mice are dying. This raises the question of whether increased bacterial growth is driving susceptibility in COX-2$^{-/-}$ mice or if increased burden is secondary to some other event, possibly increased inflammation.

Because nearly every cell can express COX-2, we sought to narrow down the cell types that required COX-2 to promote resistance. To address this, COX-2 bone marrow chimeras were generated with all possible combinations of donors and recipients. These chimeras were infected with the standard inoculum and monitored for survival up to 110 days post infection. COX-2 expression was required in both hematopoietic and nonhematopoietic cells to mediate resistance, and the most susceptible mice were COX-2$^{-/-}$ recipients with COX-2$^{-/-}$ bone marrow. Furthermore, COX-2$^{-/-}$ recipients with WT bone marrow were significantly susceptible to disease, while WT mice with COX-2$^{-/-}$ bone marrow survived for the duration of the experiment. These observations indicate nonhematopoietic COX-2 expression is essential for host survival. Nonhematopoietic cells such as epithelial cells are known to influence the host response to tuberculosis (37,38), thus it possible these cells require COX-2 expression to promote resistance.

At week 15, we examined the surviving bone marrow chimeras and discovered that the loss of COX-2 in either hematopoietic cells or non-hematopoietic cells resulted in increased bacterial burden. These mice also had increased immunopathology relative to control chimeras. These observations suggest COX-2 expression is vital in a variety of cell types during tuberculosis. Additionally, eliminating COX-2 expression in only hematopoietic or nonhematopoietic cells increased survival time and allowed us to examine much later time points than previously possible. In the end, the week 15 results with the bone marrow chimeras were the most consistent and provided the clearest differences from WT controls.

Overall, it appears that immunopathology and bacterial burden are increased when COX-2 is absent from either hematopoietic or nonhematopoietic cells. The question is whether immunopathology is driving bacterial growth. Based on the data, we cannot be certain, but it is intriguing that COX-2$^{-/-}$ mice control bacterial growth during the early stages of infection. Because bacterial growth can be controlled in the absence of COX-2, we favor the hypothesis that increased immunopathology underpins the increased susceptibility of these mice. Prostanoids can have a variety of anti-inflammatory effects (39), so it is conceivable that they regulate inflammatory responses during chronic infection. Additionally, the inhibition of COX-2 can shuttle arachidonic acid into pathways generating leuktrienes (40), thus it is possible that the absence of COX-2 leads to the accumulation of pro-inflammatory lipid mediators. Therefore, we hypothesize COX-2 expression is required during chronic infection to mediate inflammation.

Materials and Methods

Mice

BALB/c (WT), B6129SF2/J (WT), COX-1$^{-/-}$ (B6;129S6-Ptgs1^{tm1Fun}/J), and COX-2$^{-/-}$ (B6;129S-Ptgs2^{tm1Jed}/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME). COX-2$^{-/-}$ mice backcrossed on the BALB/c background for 10 generations were obtained from Laura Fredenberg (35). Mice were 6 to 10 weeks old at the start of all experiments. All animal experiments were performed in accordance with National and European Commission guidelines for the care and handling of laboratory animals and were approved by the National Veterinary Directorate and by the local Animal Ethical Committee or Institutional Animal Care and Use Committee (Animal Welfare Assurance no. A3023-01 [DFCI] or A3306-01 [UMMS]), under Public Health Service assurance of Office of Laboratory Animal Welfare guidelines). Mice infected with *M. tuberculosis* were housed in a biosafety level 3 facility under specific pathogen-free conditions at DFCI or at UMMS.

Generation of mouse bone marrow chimeras

Bone barrow chimeras were made by lethally irradiating recipients (2 doses of 500 rads separated by three hours). Bone marrow was flushed from the femurs, tibia, and humeri of donor mice and RBC lysed. Each recipient mouse received a total of 10^7 bone marrow cells via lateral tail vein injection and was kept on antibiotic-treated water for 5 weeks. Bone marrow chimeras were infected with Mtb 10 weeks after transfer of the bone marrow cells.

Experimental infection and bacterial quantification

Infection with *M. tuberculosis* (Erdman strain) was performed via the aerosol route, and mice received a day 1 inoculum of 100-200 CFU unless otherwise indicated. A bacterial aliquot was thawed, sonicated twice for 10 seconds in a cup horn sonicator, and then diluted in 0.9% NaCl–0.02% Tween 80. A 15 ml suspension of *M. tuberculosis* was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technologies) and mice were infected using a nose-only exposure unit (Intox Products). Alternatively, the bacterial aliquot was diluted in a final volume of 5ml, and mice were infected using a Glas-Col aerosol-generation device. At different times post-infection, mice were euthanized by carbon dioxide inhalation, organs were aseptically removed, individually homogenized and viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H11 agar plates. Plates were incubated at 37ºC and *M. tuberculosis* colonies were counted after 21 days. For intravenous infections, bacteria were prepared similarly and injected via the lateral tail vein. The inoculum was assessed by plating the spleens of control mice 24 hours after injection.

Tissue histopathology

Lung tissues were fixed in 10% zinc formalin and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin and eosin. Images were obtained using a microscope (DMLB; Leica) and a digital camera (DFC420; Leica). The images were adjusted and assembled in Photoshop (Adobe).

Cytokine measurements

Infected or naive lungs were perfused with 10 ml of sterile RPMI followed by digestion with collagenase. Collagenase-treated cells were passed through a 70-µm cell strainer in a total volume of 4 ml PBS 1% FCS according to the protocol of Mayer-Barber et al. (2010). After centrifugation, 100 µl of the cell-free lung suspensions was analyzed by ELISA for IFN-γ, TNF, AND IL-1β (from Biolegend, CA, USA,). Absorbance was recorded at 450 nm using an ELISA microplate reader (VersaMac; Molecular Devices).

FACS analysis

Cell suspensions from lung, spleen and lymph nodes were prepared by gentle disruption of the organs through a 70µm nylon strainer (Fisher) or using the GentleMacs apparatus (Miltenyi Biotec, Germany) according to the manufacturer instructions. For lung preparations, tissue was digested for 30-60 min at 37°C in cRPMI with 300U/ml collagenase (Sigma) prior to straining. Erythrocytes were lysed using a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM sodium EDTA pH 7.2) and, after washing, cells were resuspended in supplemented RPMI (cRPMI - 10% heat inactivated FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml streptomycin and 50 U/ml penicillin, all from Invitrogen) or MACS buffer (Miltenyi Biotec, Germany). Cells were enumerated in 4% trypan blue on a hemocytometer or using a MACSQuant flow cytometer (Miltenyi Biotec, Germany). Surface staining was performed with antibodies specific for mouse CD3 (clone 17A2), CD3ε (clone 145-2C11) CD4 (clone GK1.5), CD8 (clone 53-6.7), CD11b (clone M1/70), CD19 (clone

6D5), Gr1 (RB6-8C5), and F4/80 (BM8), (from Biolegend, CA, USA, or from BD Pharmingen, CA, USA). All staining was performed for 20 min at 4°C and, unless otherwise stated, cells were fixed before acquisition with 1% formaldehyde in PBS for 30-60 min. Cell analysis was performed on a FACS Canto (Becton Dickinson, NJ, USA) or on a MACSQuant flow cytometer (Miltenyi Biotec, Germany). Data were analyzed using FlowJo Software (Tree Star, OR, USA). For FACS analysis, single-lymphocyte events were gated by forward scatter versus height and side scatter for size and granularity.

Statistical analysis

All data are represented as mean with SEM. All comparisons of two groups were done with an unpaired student's t-test and are indicated in the figure legends. Comparisons of more than 2 groups were done by one way ANOVA followed by Dunnett's multiple comparisons. The log-rank (Mantel-Cox) test was used for statistical analysis for survival experiments. Significance was represented by the following symbols: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

References

1. Harizi H, Corcuff J-B, Gualde N. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. Trends in Molecular Medicine. 2008 Oct;14(10):461–9.

2. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014 Jun 5;510(7503):92–101.

3. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem. 2000;69:145–82.

4. Molloy A, Laochumroonvorapong P, Kaplan G. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. J Exp Med. Rockefeller Univ Press; 1994;180(4):1499–509.

5. Oddo M, Renno T, Attinger A, Bakker T, MACDONALD HR, Meylan PR. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. J Immunol. 1998 Jun 1;160(11):5448–54.

6. Martin CJ, Booty MG, Rosebrock TR, Nunes-Alves C, Desjardins DM, Keren I, et al. Efferocytosis is an innate antibacterial mechanism. Cell Host Microbe. 2012 Sep 13;12(3):289–300.

7. Park JS. Virulent clinical isolates of Mycobacterium tuberculosis grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. Journal of Leukocyte Biology. 2005 Oct 4;79(1):80–6.

8. Chen M, Gan H, Remold HG. A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. J Immunol. 2006 Mar 15;176(6):3707–16.

9. Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, et al. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. Infection and Immunity. 1997 Jan;65(1):298–304.

10. Miller JL, Velmurugan K, Cowan MJ, Briken V. The Type I NADH Dehydrogenase of Mycobacterium tuberculosis Counters Phagosomal NOX2 Activity to Inhibit TNF-α-Mediated Host Cell Apoptosis. Deretic V, editor. PLoS Pathog. 2010 Apr 22;6(4):e1000864.

11. Chen M, Divangahi M, Gan H, Shin DSJ, Hong S, Lee DM, et al. Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. Journal of Experimental Medicine. 2008 Nov

24;205(12):2791–801.

12. Divangahi M, Chen M, Gan H, Desjardins D, Hickman TT, Lee DM, et al. Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. Nat Immunol. 2009 Aug;10(8):899–906.

13. Andrews NW. Membrane repair and immunological danger. EMBO Rep. 2005 Sep;6(9):826–30.

14. Andrews NW, Almeida PE, Corrotte M. Damage control: cellular mechanisms of plasma membrane repair. Trends in Cell Biology. 2014 Dec;24(12):734–42.

15. Regan JW. EP2 and EP4 prostanoid receptor signaling. Life Sciences. 2003 Dec;74(2-3):143–53.

16. Behar SM, Martin CJ, Booty MG, Nishimura T, Zhao X, Gan H-X, et al. Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. Mucosal Immunology. Nature Publishing Group; 2011 Feb 9;:1–9.

17. Tobin DM, Ramakrishnan L. TB: the Yin and Yang of lipid mediators. Current Opinion in Pharmacology. 2013 Jul 9.

18. Bannenberg GL, Aliberti J, Hong S, Sher A, Serhan C. Exogenous pathogen and plant 15-lipoxygenase initiate endogenous lipoxin A4 biosynthesis. J Exp Med. 2004 Feb 16;199(4):515–23.

19. Vance RE, Hong S, Gronert K, Serhan CN, Mekalanos JJ. The opportunistic pathogen Pseudomonas aeruginosa carries a secretable arachidonate 15-lipoxygenase. Proc Natl Acad Sci USA. 2004 Feb 17;101(7):2135–9.

20. Divangahi M, Desjardins D, Nunes-Alves C, Remold HG, Behar SM. Eicosanoid pathways regulate adaptive immunity to Mycobacterium tuberculosis. Nat Immunol. 2010 Aug;11(8):751–8.

21. Aliberti J, Hieny S, Reis e Sousa C, Serhan CN, Sher A. Lipoxin-mediated inhibition of IL-12 production by DCs: a mechanism for regulation of microbial immunity. Nat Immunol. 2001 Dec 17;3(1):76–82.

22. Bafica A, Scanga CA, Serhan C, Machado F, White S, Sher A, et al. Host control of Mycobacterium tuberculosis is regulated by 5-lipoxygenase-dependent lipoxin production. J Clin Invest. 2005 Jun;115(6):1601–6.

23. Tobin DM, Vary JC, Ray JP, Walsh GS, Dunstan SJ, Bang ND, et al. The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. Cell. 2010 Mar 5;140(5):717–30.

24. Mayer-Barber KD, Sher A. Cytokine and lipid mediator networks in tuberculosis.

Immunol Rev. 2015 Mar;264(1):264–75.

25. Herb F, Thye T, Niemann S, Browne ENL, Chinbuah MA, Gyapong J, et al. ALOX5 variants associated with susceptibility to human pulmonary tuberculosis. Human Molecular Genetics. 2007 Dec 7;17(7):1052–60.

26. Curtis J, Kopanitsa L, Stebbings E, Speirs A, Ignatyeva O, Balabanova Y, et al. Association analysis of the LTA4H gene polymorphisms and pulmonary tuberculosis in 9115 subjects. Tuberculosis. Elsevier Ltd; 2010 Nov 26;:1–4.

27. Yang J, Chen J, Yue J, Liu L, Han M, Wang H. Relationship between human LTA4H polymorphisms and extra-pulmonary tuberculosis in an ethnic Han Chinese population in Eastern China. Tuberculosis (Edinb). 2014 Dec;94(6):657–63.

28. Idborg H, Olsson P, Leclerc P, Raouf J, Jakobsson P-J, Korotkova M. Effects of mPGES-1 deletion on eicosanoid and fatty acid profiles in mice. Prostaglandins & Other Lipid Mediators. 2013 Dec;107:18–25.

29. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, et al. Cutting Edge: Caspase-1 Independent IL-1 Production Is Critical for Host Resistance to Mycobacterium tuberculosis and Does Not Require TLR Signaling In Vivo. The Journal of Immunology. 2010 Mar 19;184(7):3326–30.

30. Mayer-Barber KD, Andrade BB, Barber DL, Hieny S, Feng CG, Caspar P, et al. Innate and Adaptive Interferons Suppress IL-1α and IL-1β Production by Distinct Pulmonary Myeloid Subsets during Mycobacterium tuberculosis Infection. Immunity. Elsevier Inc; 2011 Dec 23;35(6):1023–34.

31. Mayer-Barber KD, Andrade BB, Oland SD, Amaral EP, Barber DL, Gonzales J, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. Nature. 2014 Jul 3;511(7507):99–103.

32. Langenbach R, Loftin C, Lee C, Tiano H. Cyclooxygenase knockout mice: models for elucidating isoform-specific functions. Biochem Pharmacol. 1999 Oct 15;58(8):1237–46.

33. Chackerian AA, Behar SM. Susceptibility to Mycobacterium tuberculosis: lessons from inbred strains of mice. Tuberculosis. 2003 Oct;83(5):279–85.

34. Ejima K. Cyclooxygenase-2 deficient mice are resistant to endotoxin-induced inflammation and death. The FASEB Journal. 2003 May 8.

35. Fredenburgh LE, Suarez Velandia MM, Ma J, Olszak T, Cernadas M, Englert JA, et al. Cyclooxygenase-2 Deficiency Leads to Intestinal Barrier Dysfunction and Increased Mortality during Polymicrobial Sepsis. The Journal of Immunology. 2011 Nov 2;187(10):5255–67.

36. Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, et al. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature. 1995 Nov 23;378(6555):406–9.

37. Li Y, Wang Y, Liu X. The role of airway epithelial cells in response to mycobacteria infection. Clinical and Developmental Immunology. 2012;2012:791392.

38. Nouailles G, Dorhoi A, Koch M, Zerrahn J, Weiner J, Faé KC, et al. CXCL5-secreting pulmonary epithelial cells drive destructive neutrophilic inflammation in tuberculosis. J Clin Invest. 2014 Mar;124(3):1268–82.

39. Scher JU, Pillinger MH. The anti-inflammatory effects of prostaglandins. J Investig Med. 2009 Aug;57(6):703–8.

40. Marnett LJ. Mechanisms of cyclooxygenase-2 inhibition and cardiovascular side effects: the plot thickens. Cancer Prevention Research. 2009 Apr;2(4):288–90.

Appendix 2: Author's publications

Nunes-Alves C, **Booty MG**, Carpenter SM, Rothchild R, Desjardin D, Steblenko K, et al. Clonal T cells expansions against immunodominant epitopes are protective during tuberculosis. PLOS Pathogens 2015; *in press*.

Behar SM, Carpenter SM, **Booty MG**, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. Seminars in Immunology 2014;26:559–77.

Nunes-Alves C, **Booty MG**, Carpenter SM, Jayaraman P, Rothchild AC, Behar SM. In search of a new paradigm for protective immunity to TB. Nature Reviews Microbiology 2014;12:289–99.

Balow JE, Ryan JG, Chae JJ, **Booty MG**, Bulua A, Stone D, et al. Microarray-based gene expression profiling in patients with cryopyrin-associated periodic syndromes defines a disease-related signature and IL-1-responsive transcripts. Annals of the Rheumatic Diseases 2013;72:1064–70.

Martin CJ, **Booty MG**, Rosebrock TR, Nunes-Alves C, Desjardins DM, Keren I, et al. Efferocytosis is an innate antibacterial mechanism. Cell Host Microbe 2012;12:289–300.

Behar SM, Martin CJ, **Booty MG**, Nishimura T, Zhao X, Gan H-X, et al. Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. Mucosal Immunology 2011:1–9.

Ryan JG, Masters SL, **Booty MG**, Habal N, Alexander JD, Barham BK, et al. Clinical features and functional significance of the P369S/R408Q variant in pyrin, the familial Mediterranean fever protein. Annals of the Rheumatic Diseases 2010;69:1360–3.

Aksentijevich I, Masters SL…**Booty M**...Kastner DL, Goldbach-Mansky R. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. New England Journal of Medicine 2009;360:2426–37.

Booty MG, Chae JJ, Masters SL, Remmers EF, Barham B, Le JM, et al. Familial Mediterranean fever with a single MEFV mutation: where is the second hit? Arthritis and Rheumatism 2009;60:1851–61.

Ryan JG, de Koning HD, Beck LA, **Booty MG**, Kastner DL, Simon A. IL-1 blockade in Schnitzler syndrome: ex vivo findings correlate with clinical remission. Journal of Allergy and Clinical Immunology 2008;121:260–2.

Remmers EF, Plenge RM…**Booty MG**…Kastner DL, Gregerson PK. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. New England Journal of Medicine 2007;357:977–86.